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EVALUATION OF PROCEDURES FOR DETERMINING THE ELEMENTAL COMPOSITION OF PSEUDOMONAS CUPRODURANS

FREDERICK JAY PASSMAN

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EVALUATION OF PROCEDURES FOR DETERMINING THE ELEMENTAL COMPOSITION OF *PSEUDOMONAS CUPRODURANS*

by

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ABSTRACT

EVALUATION OF PROCEDURES FOR DETERMINING THE ELEMENTAL COMPOSITION OF PSEUDOMONAS CUPRODURANS

by

FREDERICK J. PASSMAN

Standardizing a procedure for preparing bacteria for elemental analysis would provide a means for evaluating the effects of various physicochemical parameters on bacterial elemental composition. I evaluated seven sequential processes including growth medium preparation, bacterial cultivation, harvest, washing, ashing, final preparation for analysis, and analysis for their relative contributions to error in the determination of the elemental composition of the marine bacterium Pseudomonas cuprodurans.

Preliminary experiments demonstrated that filter sterilizing the growth medium, cultivating and harvesting P. cuprodurans by continuous techniques, and washing three times in chilled double-distilled deionized water provided a contaminant-free bacterial sample. Dry-ashed bacterial samples gave precise ash yield data. Subsequent digestion in concentrated HNO_3 and dissolution in HCl provided samples in a suitable matrix for analysis by atomic

absorption spectrophotometry.

I cultivated P. cuprodurans in modified 2216E broth medium in a continuous cultivation apparatus, obtaining ten identical subsamples from each of five experiments. Physicochemical parameters among the five continuous culture experiments were: salinity, 26.5 ± 0.00 ppt; pH 7.50 ± 0.005; temperature, 30.0 ± 0.35; growth medium flow rate 3.61 ± 0.46 ml/min. Cultural parameters were: $\bar{A}_{420}$, 1.66 ± 0.25; specific growth rate μ', 0.21 ± 0.026; dry weight yield (mg/l), Y_{dw}, 233 ± 30.0. The mean ash yield was 7.99 ± 0.483 % (dry weight). Major protoplasmic elements as % dry wt were: C, 52.1 ± 1.02; H, 7.36 ± 0.195; N, 14.3 ± 0.21; S, 0.51 ± 0.012. O was 17.7 % (dry wt) by subtraction. Other elements determined were (ppm dry wt): P, 17500 ± 1980; Mg, 4000 ± 1370; Na, 2800 ± 1140; Ca, 2600 ± 2550; K, 2150 ± 708; Fe 170 ± 55.9; Zn, 97.0 ± 33.8; Cu 27.4 ± 7.01. Interexperimental variation ranged from 1.46 % for N to 99.6 % for Ca. Variation for major protoplasmic elements and minor ash constituents was substantially less than for major ash constituents. Intraexperimental variation was sufficiently low to permit resolution of ash yield and elemental composition differences among experiments.

INTRODUCTION

Early determinations of the elemental composition of bacteria involved colorimetric (Dawson, 1919) or isotope dilution techniques (Roberts et al., 1955). Although investigators from other disciplines (Struempler, 1975, atmospheric particulates; Knauer and Martin, 1973, zooplankton; Fuge and James, 1973, brown algae) have utilized modern instrumental techniques including atomic absorption spectrophotometry, neutron activation analysis and optical emission spectrography to obtain multi-elemental analysis, bacteriologists have relied on the limited data acquired originally more than three decades ago.

As anlytical instrumentation has become more refined, detection limits have decreased to > 1 ppb to $\sim$ 1 ppm depending on the technique and element (Willard, Merritt and Dean, 1974). Consequently, the need for identifying sources of contamination and unintentional experimentally introduced procedural variations has become more critical (Hamilton et al., 1972). Addressing this issue, I identified seven successive processes involved in achieving elemental analyses of bacteria. The processes included bacterial cultivation, harvest, washing, ashing, final preparation for analysis, and final analysis. I evaluated each of the processes, developed a series of standard procedures and determined the overall precision of the technique.

LITERATURE REVIEW

Vinogradov (1953), Stiles (1961) and Sanchelli (1969) have reviewed the history of trace element analysis. In the same monograph, Vinogradov (1953) compiled an exhaustive tabulation of the elemental composition of marine organisms representing all taxonomic levels from Prokaryota to Mammalia. More recently, Bowen (1966) surveyed the biogeochemistry, distribution and biochemistry of trace elements. Zajic (1969) and Williams (1971) have both surveyed the biological role of trace elements. I considered these topics to be beyond the scope of this discussion, and have limited this review to a consideration of current investigations of the elemental composition of bacteria, marine organisms and their environment.

Dulka and Risby (1976) summarized data on the roles of elements in biological systems, sources and magnitudes of anthroponomic pollutants, and analytical approaches used to investigate the roles of trace elements in the environment and in biological systems. They cited the need to develop procedures for baseline data accumulation and toxicological research, concluding that current knowledge regarding metal-biological system interactions was minimal.

Redfield (1942) was one of the earliest investigators to establish a relationship between elemental concentrations and metabolic activity in the ocean.

Refining his earlier observations, Redfield (1958) presented a model for the biochemical cycle controlling C, H, P, and S concentrations in the ocean. Redfield developed his model by comparing oceanic C, N, and P concentrations with planktonic concentrations, finding a quantitative relationship between N and P concentrations in both systems. Later, Redfield, Ketchum, and Richards (1963) further refined the model, and related it to oceanic circulation patterns. In this final paper, they reported that under certain circumstances, Fe, Mn, Cu, Zn, Co, or Mb might have been growth limiting factors. Kench (1961) focused on the relationship between metal ions and biological systems.

Discussing diagenetic processes in a reducing environment, Nissenbaum (1972) found support for Redfield's model. Nissenbaum cited the role of biological sulfate reduction in maintaining the pH and ionic balance in the hypolimnion of a British Columbian fjord. Nissenbaum (1975) had more difficulty explaining the biogeochemistry of the Dead Sea in terms of Redfield's model. He speculated that in the Dead Sea, trace metal concentrations were sufficiently high (ppm: Cu, 300; Zn, 500; Mn, 4000; Fe, 15; Ni, 25; Co, 8; Cd, 10; Pb, 300; Ce, 2.5) to inhibit microbial productivity. Bryan (1971) and Clarke (1974), reviewing the effects of heavy metals on marine organisms, presented sufficient data from various sources to support Nissenbaum's hypothesis.

Dulka and Risby (1976) acknowledged the fact that most current theories regarding the interactions between

the lithosphere and biosphere were based on few elemental investigations. The concentrations of most elements in seawater were sufficiently low (< 1 ppb) to be undetectable unless they were pre-concentrated prior to analysis (Goldberg, 1965). Greenhalgh, Riley and Tongudai (1966) introduced a procedure, utilizing Amberlite GC 120 to concentrate the major cations from seawater. Using another ion exchange resin -- Chelex 100 -- Riley and Taylor (1968a,b) successfully extracted Al, As, Ba, Bi, Cd, Cs, Ce, Cr, Co, Cu, I, Pb, Mn, Hg, Mo, Ni, P, Re, Sc, Se, Ag, Tl, Th, Sn, W, U, V, Y and Zn from seawater for analysis.

A number of investigators preferred solvent extraction to chelation on resin columns (Slavin, 1968). Brooks, Presley, and Kaplan (1967) developed an ammonium pyrrolidine-carbodithioate (APCD) methylisobutyl ketone (MIBK) system for simultaneously extracting Co, Cu, Fe, Pb, Ni and Zn from seawater. Stolzberg (1975) determined the relationship between pH and efficiency of extraction and made a number of recommendations for optimizing the APCD/MIBK solvent extraction system. Nix and Goodwin (1970) adapted the technique to fresh water analysis by substituting diethyldithiocarbamate (DDC) for APCD. They extracted Co, Ni, Cr, Pb and Zn from reservoirs and lake waters. Krishnaswami et al. (1972) discovered that they could quantitatively recover Si, Ra, Th, and Pb from seawater using acrilan fiber preloaded with ferric hydroxide.

Paulsen, Smith and Mark (1975) reviewed the

criteria for developing an in situ trace element sampler. Davey and Soper (1975) designed an in situ sampling apparatus comprised of Chelex 100 resin columns in series with four 1.0 liter collection bottles. Their apparatus collected particulate and dissolved Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn from seawater with minimal contamination.

With the implementation of the International Decade of Oceanic Exploration, and the development of the procedure introduced above, data acquisition had increased during the past decade (Dulka and Risby, 1976). Spencer and Brewer (1969) investigated Cu, Zn and Ni seasonal variations and distributions in the Gulf of Maine and the Sargasso Sea. Ni and Zn concentrations increased with depth and they postulated that surface organisms concentrated these elements very slowly. Cu, Ni and Zn concentrations in surface waters did not vary seasonally. Bender and Gagner (1976) confirmed these observations. In contrast, Morris (1971) observed a direct correlation between particulate Zn and Cu and phytoplankton blooms in the Menai Straits.

During a series of Geosecs cruises in 1970, investigators found no correlation between ^{228}Ra, ^{3}H, Ba or Sr, and biological activity (Trier, Broeker and Feeley, 1972; Roether and Münnich, 1972; Wolgemath and Broeker, 1970; Bernat, Church and Allegre, 1972). Moore (1972) found ^{228}Ra useful for investigating water exchange rates across the main thermocline.

Abdullah, Royle and Morris (1972) correlated Cu,

Pb, Cd and Zn concentrations in regions of Cardigan Bay and Liverpool Bay to industrial effluents. Butterworth, Lester and Nickless (1972) arrived at similar conclusions concerning trace metal distributions in the Severn Estuary around Bristol. Investigating trace metal distributions in surface waters of the world's oceans, Chester and Stoner (1974b) observed two types of distribution patterns. Zn, Cd, and Cu concentrations did not differ between near shore waters and open ocean waters. Ni and Mn concentrations were lower in open ocean waters than in near shore waters. Among samples, trace metal concentrations in near shore waters varied substantially more than did concentrations in oceanic samples. Chester and Stoner (1974b) reported the following base line oceanic concentrations (μg/liter): Zn, 1.4; Ni, 1.2; Mn, 0.22; Cd, 0.07; Cu, 0.8.

Turekian and Imbrie (1966) determined Ba, Co, Cu, Ni, Pb, Cr, Mn, and Sn in deep-sea cores from the Atlantic Ocean. For the most part, trace metal concentrations were not related to either clay mineralogy or water depth. Mn, Ni, and Co were more concentrated in areas of low clay accumulation. Metal concentrations were elevated in near-shore sediments relative to sediments from the open ocean (Preston et al., 1972).

Analyzing soil-sized particulates in the ocean, Chester and Stoner (1974a) reported that Mn, Ni, Co, Ga, Cr, V, Ba, and Sr concentrations in the particles were of the same order of magnitude as in average crustal material. Pb, Sn and Zn were concentrated by an order of magnitude

compared with crustal abundances. Regional variations in particulate Pb, Sn and Zn concentrations suggested that these elements reflected industrial or domestic pollution.

Ramamoorthy and Kushner (1975b) discovered that in some rivers, HCO_3^- and $CO_3^=$ bound significant concentrations of heavy metals; however, small organic molecules such as fulvic acid were more important in other river systems. Duinker and Nolting (1976) developed a model for the distribution of particulate trace metals in the Rhine Estuary. They noted correlations between Fe and Mn concentrations, and Cu and Zn concentrations in particles. Near shore, particles were enriched with Fe and Mn, and offshore they were enriched with Cu and Zn. Duniker and Nolting concluded that river-borne chelation reactions precipitated dissolved Mn and Fe during the early stages of mixing.

A number of mechanisms affected trace metal transport. Feely (1975) investigated particulate trace element concentrations in the nepheloid layer just above the sea floor. Wallace and Duce (1975) used an adsorptive bubble separation technique to examine particulate organic carbon and particulate trace metal transport at the sea-air interface. Other investigators have analyzed for particulate trace metals in industrial pollutants (Von Lehman, Jangers, and Lee, 1974), and in dust, rain and snow (Struempler, 1975, 1976; Robinson, 1976).

Analyses of particulate trace metals in various systems had provided a perspective on the global distribution

of anthropogenic pollutants (Chester and Stoner, 1974a). Point source pollutant discharges caused dramatic local enrichment of Cu, Pb, Cd and Zn (Butterworth et al., 1972) and atmospheric effluents carried particulate trace metals vast distances (Struempler, 1975).

In 1959, Nicholls, Curl and Bowen reported on the elemental analysis of ten zooplankton species. They observed that some species actively concentrated B, and others excluded it. They also speculated that pteropod tests trapped V and Pb removing these elements from the water column during sedimentation. A decade later, while analyzing brown algae for Sr, Ca and Mg, Haug and Smidsrod (1967) proposed a model to interpret the relative concentrations of these three elements in the algae. They concluded that the algae served as a matrix containing glucuronic acid enriched alginates. Ca, Mg and Sr in the alginates were in ion exchange equilibria with the seawater and were proportional to the alginate's uronyl residue content (Haug and Smidsrod, 1967).

Cherry, Gericke, and Shannon (1969) determined ^{228}Th in zooplankton and phytoplankton from seven South Atlantic water masses. ^{228}Th in seawater was barely at the detection level, but was approximately 2.7×10^{-19} g/g wet zooplankton and 6.5×10^{-19} g/g wet phytoplankton. Despite inconclusive data, Cherry et al surmised that ^{228}Th concentrations in plankton from different water masses differed significantly and could be used as an oceanic tracer. Conducting an analogous investigation with ^{226}Ra,

Shannon and Cherry (1971) demonstrated that plankton in the Aquthas Current contained 7.7 x 10^{-12} g ^{226}Ra/g dw; but plankton from other regions contained only 1.0 x 10^{-12} g ^{226}R/g dw.

Martin and Knauer (1973) and Knauer and Martin (1972) performed multielemental analysis of plankton collected along a transect between California and Hawaii and along the California-Oregon coast. They grouped their phytoplankton samples according to elemental composition. Samples containing < 5.7 ppm Ti contained 49-380 ppm in Fe. A second group of samples contained 6 - 137 ppm Ti and 446 - 2970 ppm Fe. The third group lacked Ti (< 5.7 ppm), but concentrated Sr (384-3934 ppm as compared with 53-260 ppm in the other groups). Martin and Knauer summarized their plankton analyses as shown in Table 1.

To determine whether biological speciation affected elemental composition, Riley and Roth (1971) analyzed 15 phytoplankton species which they had grown under controlled conditions in defined medium. Compositional variations among individuals within a species obfuscated any variation among species. Cowgill and Burns (1975) compared two Daphnia species with some fresh water algae on which the Daphnia grazed. Daphnia concentrated Cl, Ca and Na by factors of 17.5, 6.9 and 4.5 over levels found in algae. The algae concentrated other elements more than the Daphnia did. Concentration factors were: Mo, 13.4; Fe, 12.2; Mg, 10.6; Sn, 8.1; Ag, 7.5; Ti, 6.1; Be, 3.7; Cu, 3.0;

Table 1. Median values for groups of plankton in samples collected off the coasts of California, Hawaii and Oregon (Martin and Knauer, 1973).

µg/g dry weight

	Monterey Bay			Hawaii		Oregon	
						Microplankton	
Element	Euphausids n = 9	Copepods n = 10	Radiolarians n = 6	Zooplankton n = 1	Microplankton n = 4	No. 007 n = 1	No. 006 n = 1
Pb	2.1	3.3	2.1	[2]2.1	31.0	27.7	4.3
Hg	0.08	0.11	0.14	0.11	0.34	0.71	0.48
Cd	2.8	4.1	6.4	2.3	1.6	1.1	0.4
Ag	<0.1	<0.1	<0.1	0.26	0.17	0.24	0.12
Ni	3.8	2.0	3.7	[3]8.4	11.6	5.7	2.0
Mn	3.6	4.4	6.4	4.3	15.0	7.4	5.2
Cu	15.6	10.5	6.5	11.5	57.5	45.8	24.1
Fe	92	197	315	[2]100	1985	745	290
Zn	69	113	110	[2]180	780	970	163
Al	31	70	137	15	102	25	17
Ba	24	17	17	26	59	314	323
Sr	163	125	163	720	8,900	1,800	3,600
K	12,300	12,000	11,000	10,000	7,900	12,900	6,900
Ca	9,000	7,770	8,500	20,500	12,000	7,000	6,500
Mg	8,000	8,600	11,200	8,900	13,300	18,000	17,300
Na	56,100	74,300	103,300	80,000	115,000	133,100	111,300

Notes:
1) n = 9 for Pb, Fe, Ni and Zn; n = 14 for remaining elements
2) Medians determined after metal particles were removed
3) Median determined with Nos. 582, 583, 588-590 omitted

Zn, 2.7; I, 2.5; Mn, 2.3; P, 1.9; Al, 1.7; As, 1.6; Co, 1.7; K, 1.5; Pb, 1.3; Hg, 1.4; Si, 1.1. Their data suggested that although species differences may not have been significant, differences at higher taxonomic echelons were.

Knauer and Martin (1973) monitored Cd, Cu, Mn, Pb, and Zn in water and phytoplankton in Monterey Bay. Average trace metal concentrations reflected hydrographical conditons rather than seasonal variation. For example, Cd concentrations in intense upwelling, low upwelling, oceanic and mixed waters were 0.30, 0.11, 0.03, and 0.09 µg/liter respectively (Knauer and Martin, 1973). They observed similar trends for the other elements. On a transect between Hawaii and Monterey, oceanic surface water concentrations (µg/liter) for the elements averaged: Cd, 0.02; Cu, 0.08 $\pm$ 0.2; Mn, 0.2 $\pm$ 0.08; Zn, 2.0 $\pm$ 0.7; Pb < 0.2. In contrast to the water samples, planktonic trace metal concentrations varied seasonally. Knauer and Martin (1973) found maximal concentrations during late spring and early autumn (Cd, 7 ppm; Cu, 40 ppm; Mn, 30 ppm; Zn, 700 ppm; Pb 35 ppm) and minimal concentrations in mid-summer (Cd, Cu, Mn, Zn and Pb all 1-5 ppm).

Fuge and James (1973), monitoring Zn, Cd, Ca, Mn, Fe, Co and Mo in Fucus species also observed seasonal variations in elemental composition (Table 2). All of the elements demonstrated similar seasonal trends, being concentrated most during the spring and least during the autumn. Comparing trace element concentrations in Fucus vesiculosis at 11 stations, Fuge and James (1973) observed

Table 2. Fucus serratus, seasonal variation in trace element concentrations (ppm dry weight) at Aberystwyth (A) and Nefyn (N), $\bar{X}$ = mean; SD = standard deviation; N = 30, except Nefyn, June 69 (N = 29) (Fuge and James, 1973).

		June 69		Sept. 69		Jan. 70		March 70		June 70		Sept. 70		Feb. 71	
		$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Zn	A	377.0	47.6	266.0	40.1			613.0	31.9	296.0	39.1	443.0	60.6	602.0	19.3
	N	76.0	6.1	56.0	5.1	113.0	6.0								
Cu	A	4.44	0.77	2.48	0.23			4.20	0.58	3.30	0.61	4.14	0.68	4.70	0.59
	N	3.05	0.25	2.45	0.34	4.80	1.09	5.45	0.72	3.42	0.39	4.90	0.46	5.24	0.67
Mn	A	173.0	22.7	136.0	17.5			110.0	8.45	114.0	14.6	86.0	4.8	137.0	10.0
	N	123.0	10.2	148.0	12.1	133.0	8.3	133.0	10.9	76.0	9.2	116.0	7.8	134.0	9.5
Fe	A	131.0	35.5	78.0	10.5			146.0	15.6	135.0	15.4	112.0	8.0	146.0	11.0
	N	73.0	7.3	79.0	7.0	97.0	5.8	103.0	6.9	86.0	11.1	84.0	10.7	197.0	18.4
Ni	A	11.4	1.04	7.8	0.97			19.9	2.35	11.6	1.79	13.0	2.09	9.5	1.68
	N	10.7	0.88	9.0	1.47	17.9	1.67	18.5	1.48	13.0	1.94	12.3	2.46	15.6	2.03
Co	A	5.0	1.2	5.0	0.6			7.0	1.2	8.0	1.3	7.0	1.5	8.0	1.6
	N	4.0	0.4	4.0	1.8	8.0	1.2	10.5	1.8	7.0	1.3	7.0	1.4	9.5	1.7
Cd	A	3.19	0.39	1.89	0.17			3.18	0.32	2.48	0.27	4.43	1.01	4.91	0.78
	N	1.16	0.50	2.15	0.32	2.76	0.29	3.65	0.39	4.00	0.27	2.32	0.27	6.73	1.15
Mo	A	0.40	0.13	0.31	0.08			0.42	0.20	0.35	0.06	0.49	0.17	0.22	0.04
	N	0.25	0.05	0.22	0.03	0.31	0.08	0.31	0.06	0.44	0.11	0.31	0.12	0.24	0.03
Ash %	A	16.0	0.57	14.8	0.46			19.0	0.40	11.1	0.72	17.6	0.51	16.9	0.44
	N	13.8	0.55	12.8	0.52	20.0	0.93	17.2	1.25	11.8	1.10	10.2	0.39	16.6	0.40

distinct regional differences (Zn, 57 ± 2.9 - 2530 ± 201; Cu, 3.65 ± 0.63 - 25.5 ± 2.3; Mn 63 ± 6.6 - 799 ± 110; Fe, 97 ± 25.8 - 2400 ± 147; Ni, 10.8 ± 1.23 - 14.9 ± 1.76; Co, 3.5 ± 1.4 - 16.0 ± 2.3; Cd, 2.05 ± 0.30 - 31.4 ± 8.27; Mo 0.20 ± 0.04 - 1.63 ± 0.41; data in ppm dry weight, n = 30 for each value). Fucus harvested from harbors had the highest metal concentrations.

In a similar study, Whyte and Englar (1974, 1975) analyzed Nerocystis luetkeana at monthly intervals. Among 20 elements determined, Ca and P concentrations increased systematically over the course of the growing season. The composition of the stipes differed from that of the fronds (Table 3). These data substantiated earlier observations of Bryan and Hummerstone (1973), who correlated trace metal concentrations in Fucus vesiculosis with the degree of metal input from different drainage areas. Bryan and Hummerstone concluded that for long term comparative analyses, cleaned, older portions of the Fucus thallus harvested during a fixed time of year from a pre-determined location in the inter-tidal zone, provided the most satisfactory samples. Preston, et al. (1972), and Morris and Bale (1975) arrived at similar conclusions to those presented above.

Huag, Melsom and Omarg (1974) compared Zn, Cu, Pb, Cd and Hg concentrations in Ascophyllum nodosum from four regions of Hardanger Fjord, Norway. The data, presented in Tables 4a and b, demonstrated that algae could be used as pollution indicators.

Table 3. Elemental composition of Nerocystics leutkeana (from Whyte and Englar, 1974, 1975).

Element	Concentration Range[1]	
	Fronds	Stipes
Cl	12.7 - 17.0	15.1 - 26.9
K	9.65 - 11.7	17.3 - 22.3
Na	5.79 - 7.61	3.83 - 5.71
Mg	0.71 - 0.99	0.49 - 0.63
Ca	0.53 - 1.53	0.41 - 0.92
P	0.22 - 0.52	0.09 - 0.28
I	549 - 1423	826 - 1548
Sr	623 - 1030	482 - 907
B	53 - 122	44 - 135
Fe	40 - 112	10 - 93
Al	30 - 108	20 - 105
Zn	40 - 60	2 - 10
Ba	6 - 30	4 - 7
Mn	4 - 15	< 4
Cr	< 6	< 6
Cu	< 2	< 2
Cd	2.0	1.3
Pb	0.9	0.6
Co	< 0.2	< 0.2
Hg	< 0.05	< 0.05
$(\frac{\text{ash weight}}{\text{dry weight}}) \times 100$	5.89 - 9.63	7.52 - 9.60

Notes:

1) Concentration for Al, K, Na, Mg, Ca, and P are % dry weight, all others are ppm dry weight.

Table 4a. Trace metal content of Ascophyllum nodosum from Hardanger Fjordppm in dry matter (Haug et al., 1974).

	Metal	Range	Average
Area I			
4 Samples, 20 Sept. 1971	Zn	2300-3700	3220
27 Oct. 1971	Cu	85-160	111
	Pb	70-95	81
	Cd	12-16	14.3
	Hg	4-20	11.8
Area II			
7 Samples, 20 Sept. 1971	Zn	1700-2600	2140
27 Oct. 1971	Cu	25-50	29
	Pb	15-33	22.4
	Cd	8-11	7.3
	Hg	1.4-3	2.3
Area III			
4 Samples, 27 Oct. 1971	Zn	1750-2500	2140
20 March 1972	Cu	5-20	15
	Pb	< 3-9	5
	Cd	5-8	6.4
	Hg	0.6-1.2	1.0
Area IV			
20 Samples, 20 March 1972	Zn	1030-2150	1420
	Cu	3-11	6
	Pb	< 3	< 3
	Cd	2-4	3.5
	Hg	0.2-0.5	0.33
Area V			
6 Samples, 17 July 1972	Zn	240-370	315
	Cu	3-6	4.5
	Pb	< 3	< 3
	Cd	0.7-1.9	1.2
	Hg	0.05-0.12	0.08

Table 4b. Trace metal content of Ascophyllum nodosum from Hardanger Fjord expressed as percentage of the content in area I.

	Zn	Cu	Pb	Cd	Hg
Area I	100	100	100	100	100
Area II	66.5	26	28	51	19.5
Area III	66.5	13.5	7.0	45	8.5
Area IV	44	5.4	< 3.7	24.5	2.8
Area V	9.8	4.0	< 3.7	7.0	0.7
'Normal'	2.5	4.5	< 3.7	< 7.0	0.7

Investigators have also established relationships between trace elements in representatives of the animal kingdom and concentrations in the water column (Fowler and Oregiono, 1976). Goldberg (1957, citing Bowen and Sutton) reported that sponges concentrated Cu (1400 x), Ni (420 x), Co (50 x) and Ca (3.5 x) from seawater and excluded Mg (0.07 x). Riley and Segar (1970) reported that elemental composition varied among echinoderm species and among tissues within a specimem (Table 5). Coughtrey and Martin (1976) observed similar distribution phenomena in tissues of the pulmonate mollusc, Helix aspersa.

Developing a model for trace element transport between surface waters and the benthos, Bertine and Goldberg (1972) considered the role of molting. Fe, Co, Zn, Se, Ag and Hg in shrimp carapaces were concentrated 8×10^3 to 1×10^5 over levels in seawater (Table 6).

Bertine and Goldberg (1972) surmised that molted carapaces did mediate trace element transport through the water column. Turekian, Katz, and Chan (1973) analyzed pteropod tests for 11 elements, obtaining data which supported Bertine and Goldberg's (1972) transport model. Further evidence presented by Kustin and Toppen (1975) also supported the transport model.

The role of trace metals as environmental pollutants has provided much of the impetus for trace metal analysis in various systems (Bryan and Hummerstone, 1973). Many studies have concentrated in evaluating heavy metal distributions in populations indigenous to regions where pollution

Table 5. Elementary composition of some Echinoderms (Riley and Segar, 1970)

(Nd = not determined. All concentrations as ppm in dried (60° C) specimen).

Element	*Echinus esculentus* Oral shell	*Echinus esculentus* Aboral shell	*Echinus esculentus* Aristotle's lantern	*Echinus esculentus* Spines	*Spatangus purpureus* Test and Spines	*Echinus esculentus* Intestines	*Echinus esculentus* Gonad	*Spatangus purpureus* Gonad
Fe	6.9	2.5	1.6	16	14	22,000	15	830
Mn	56	57	88	4.5	27	11	0.52	82
Co	< 0.27	< 0.16	< 0.16	< 0.17	< 0.16	< 1.1	0.40	0.50
Ni	< 0.23	< 0.14	< 0.13	1.6	< 0.12	< 0.77	7.7	1.4
Cd	0.67	0.30	0.20	0.66	0.14	8.9	0.65	1.1
Cu	1.8	0.90	0.42	1.6	1.2	5.9	16	8.7
Pb	< 0.62	< 0.62	< 0.62	< 0.58	< 0.54	< 3.0	< 0.68	< 0.84
Zn	110	12	22	28	35	550	110	170
Ag	0.09	< 0.05	0.53	< 0.03	< 0.03	< 0.75	< 0.03	< 0.04
Cr	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Al	120	140	160	130	160	2,100	< 95	1,000
Na	7,200	11,000	8,100	10,000	8,900	52,000	27,000	35,000
K	360	460	330	650	490	9,600	7,000	5,000
Ca	300,000	300,000	300,000	320,000	290,000	40,000	2,200	11,000
Mg	16,000	15,000	18,000	10,000	21,000	10,000	4,900	5,600
Sr	930	820	920	760	1,100	150	20	< 19
P	65	78	69	64	74	2,600	8,500	2,600

Table 6. Elemental concentration factors for shrimp (Ensis ensis) carapaces and the shrimp tissues over seawater on a relative basis (dry weight concentrations in organisms vs. seawater concentrations) (Bertine and Goldberg, 1972).

Element	Carapace concentration (ppm dry weight)	Tissue concentration (ppm dry weight)	Body burden (ng) Carapace	Body burden (ng) Tissue	Body Burden in Carapace (%)	Seawater (ppm)	concentration factor Carapace	concentration factor Tissue
Rb	3.2	4.8				0.12	27	40
Fe	32	62	6.6	0.51	87	0.003	11,000	21,000
Co	0.47	0.46	0.10	0.04	75	0.00002	24,000	23,000
Sb	0.03	0.15	0.006	0.010	43	0.0003	100	500
Zn	76	39	14.8	3.9	79	0.01	7,600	3,900
Se	5.0	6.1	0.71	0.41	61	0.00009	56,000	68,000
Ag	1.1	0.24	0.22	0.02	91	∿ 0.00001	111,000	24,000
Hg	1.3	1.3	0.30	0.14	68	0.00003	43,000	43,000

problems were critical (Jensen, Rystad and Melson, 1974; Kacprzak and Chvojka, 1976). Unfortunately, these were mostly a posteriori investigations and provided little insight to biogeochemical processes in non-polluted ecosystems.

A number of investigations have been performed to determine the effect of heavy metals on bacteria. Sadler and Trudinger (1967) argued that metal toxicity was proportional to the stability of chelates formed by the metals. Toxicity to E. coli and chelate stability followed the order: Hg > Cd > Pb > Cu > Ni > Co > Zn, Fe > Mn. Jones (1967a), investigating the effect of heavy metals on E. coli survival in seawater, reported a similar toxicity trend: Cu > Ni and Zn. Reviewing the parameters determining the fate of fresh water bacteria in the sea, Jones (1971) concluded that high concentrations of inorganic salts and heavy metal ions were at least partly responsible for the rapid die-off of E. coli in seawater. He pursued this line of argument (Jones, 1973; Jones and Cobet, 1975) demonstrating that heavy metal content and low nutrient concentration were the primary factors mediating E. coli die-off in the open sea. Recently, Yang and Ehrlich (1976) observed the following toxicities to bacteria isolated from ferro-manganese nodules and deep sea sediments (grown in medium containing 0.025 % peptone: Cu (5 ppm) > Ni (10 ppm) > Co (50 ppm) > Mn (> 200 ppm). (Figures in parenthesis represented minimum inhibitory metal concentration).

During their investigations, Sadler and Trudinger (1967) noted that two Pseudomonas isolates exhibited a specific response to Cu stress. Roche (1966) pursuing a line of investigation initiated by Waksman, Johnstone and Carey (1943) isolated a number of marine bacteria resistant to 2.5×10^{-3} M Cu. McCarthy (1971) continued the investigation using P. cuprodurans, one of Roche's isolates. He reported the physiological effects of Cu on P. cuprodurans.

During the same time period, Wirsen (1966) surveyed the response of marine and fresh-water isolates to Ni stress. Cobet (1968), concentrating on one of Wirsen's isolates (Arthrobacter marinus), observed a morphological response to Ni stress. A. marinus retained normal morphology in 1×10^{-4} M Ni in 0.05 % peptone and yeast extract and 26 ppt seawater medium. At 4×10^{-4} M Ni, A. marinus became megalomorphic, attaining dimensions 5-15 times its normal length. Growth ceased at 5×10^{-4} Ni under specified growth conditions (Cobet, Wirsen, and Jones, 1970).

Groves, Wilson and Young (1974) demonstrated that 9.2×10^{-8} and 5.5×10^{-7} M Hg inhibited DNA mediated transformation of B. subtilis 168 grown on Penassay broth (Difco) during early (DNAase sensitive) and late (DNAase insensitive) phases. Doyle, Marshall and Pfander (1975) reported that among cultures of E. coli, Bacillus cereus, Lactobaillus acidophilus, Staphylococcus aureus, Streptococcus fecalis and Actinomyces niger grown on

brain heart infusion broth only E. coli and B. cereus grew in 40 µg Cd/ml. They suggested that the resistant cultures immobilized the Cd at the cell wall. However, Mitra et al. (1975) observed E. coli temporarily developing large intracellular vacuoles in response to 3×10^{-6} M Cd in glucose salts medium. They proposed that resistant E. coli excluded Cd from the cytoplasm by envacuolization. Cd distribution in resistant bacteria was: cell wall, 55 %; plasma membrane, 13 %; cytoplasm, 31 %; as compared with 2, 75 and 23 % for non-resistant members of the same population. Silver, Schottel and Weise (1976) suggested that plasmids provided underlying control of these resistance mechanisms.

In addition to their utility for developing heavy metal toxicity models, bacteria played an important role in the biogeochemical cycling of trace metals (Bowen, 1966). Investigating the deposition of Zn and Cd by marine bacteria, McLerran and Holmes (1974) reported that 85 % of the ^{65}Zn and 70 % of the ^{109}Cd added to mixed bacterial cultures was removed from solution within 120 h. After the incubation period, 20 % of the removed Zn and Cd was associated with bacterial cells, the balance being found in the precipitate. Curves of Cd and Zn removed from solution paralleled bacterial growth curves. McLerran and Holmes (1974) surmized that bacterial metabolites played a significant role in removing the heavy metals from the water column. This theory had been advanced earlier by Perlman (1965) and Jones (1970).

Zajic (1969) and Doetsch and Cook (1973) have reviewed other biogeochemical processes mediated by bacteria. Brinkman, Iverson and Blair (1976) suggested combining gas chromatography and atomic absorption spectrophotometry to study microbe-mediated metal transformations. Holm and Cox (1975) reported on the bacterial transformations of Hg, and Sayler, Nelson and Colwell (1975) discussed the role of bacteria in mediating Hg accumulation by the oyster Crassostrea virginica. Doran and Alexander (1977) have investigated microbial transformations of Se.

One area that has received little attention from investigators is multielemental analysis of bacteria. Two of the most frequently cited references listing bacterial composition (Luria, 1960; Porter, 1946) were compilations of earlier investigations. Luria (1960) cited data obtained by Roberts et al. (1955) in his tabulation of the elemental composition of E. coli. Roberts et al. monitored isotope incorporation ($^{35}SO_4^=$, ^{14}C-glucose, $CH_3{}^{14}COONa$, $^{32}PO_4^-$, and $^{14}CO_2$) and determined the major elements in the fixed salt (not extracted by water after killing cells) and free salt (extracted by water after killing) fractions (Table 7).

Dawson (1919) used colorimetric and gravimetric techniques to determine S, P (P_2O_5), Ca (as CaO) and N in E. coli. He obtained average values: 5 % Ash, 0.09 % S, 2 % P, 2 % Ca, and 4 % N (as fractions of dry weight). These values were much lower than those reported by Roberts et al. (1955).

Table 7. Elementary composition of E. coli (Roberts et al., 1955).

a. Major Constituents

Element	% Dry Weight
C	50
N	10.3 - 15
P	3.2
S	1.1
Ash (total)	12.75
Fixed Salts	7.25
Free Salts	5.5

b. Minor constituents

Element	% Fixed Salt Fraction	% Free Salt Fraction
Na	2.6	19.8
K	12.9	9.9
Ca (CaO)	9.1	13.8
Mg (MgO)	5.9	2.0
P (P_2O_5)	45.8	41.3
S (SO_4^{2-})	1.8	4.4
Cl	0.0	7.4
Fe (Fe_2SO_3)	3.4	trace
Mn	20 ppm	
Cu	80 ppm	
Al	100 ppm	

Reviewing the chemical composition of bacteria, Porter (1946) included Dawson's (1919) data for E. coli, and Curran's (1943, cited by Porter, 1946) data for some Bacillus species and Clostridium sporogenes (Table 8).

Recently, Jones (Jones, Royle and Murray, 1976; Jones, Royle and Murray 1977a,b,c (all prepared for publication)) has undertaken a series of investigations to determine the extent of trace metal assimilation by marine bacteria and the elemental composition of a number of bacterial species. Jones compared the composition of A. marinus and P. cuprodurans with the composition of the growth medium (Table 9). He also analyzed 23 eubacterial species (Table 10) and 5 cyanobacterial species (Table 11).

Kung, Raymond and Glaser, (1976) monitored E. coli metal content as a function of cell age. Measuring ion content in ng/ml, King et al. (1976) observed Zn increase from 2 to 12, closely paralleling E. coli's two step growth curve. Cellular K, Mg and Ca concentrations also increased ($\frac{\text{ng/ml at 0 min}}{\text{ng/ml at 120 min}}$: K, 150/800; Mg 40/150; Ca 30/100) during growth. The fact that elemental composition varied during growth and depended on the growth medium composition (Jones et al. 1977) seemed to substantiate Dawson's (1919) early observation:

> "To produce a determinable change in either properties or composition of bacteria, it is necessary to change their environment. This is easily done by first altering the nature of the medium upon which they are grown. By regulating the transplants with sufficient frequency to make sure that the changes observed are not due to bacterial 'old age', and by

Table 8. Elementary inorganic composition of vegetative and spore cells as determined by spectrographic analysis (Porter, 1946).

Organism	Percentage of Dry Weight								Parts per Million of Dry Weight									
	K		Ca		P		Mg		Fe		Al		Cu		Mn		B	
	Veg.[1]	Sp.[1]	Veg.	Sp.	Veg.	Sp.	Veg.	Sp.	Veg.	Sp.	Veg.	Sp.	Veg.	Sp.	Veg.	Sp.	Veg.	Sp.
Bacillus coherens	2.0	0.5	0.2	2.7	4.5	1.7	0.9	0.5	506	120	90	300	50	480	40	80	5	10
Bacillus cereus	1.1	0.2	0.2	1.9	1.6	0.9	0.2	0.3	60	170	30	430	20	150	20	30	..	..
Bacillus subtilis	1.3	0.9	0.8	1.6	3.1	1.8	1.1	0.5	100	120	100	90	80	90	20	60	5	10
Bacillus macerans	0.1	0.1	0.1	1.9	6.1	0.6	0.6	0.8	140	220	390	230	50	260	30	40	5	20
1503[2]	0.8	0.1	1.0	1.2	1.4	1.1	0.7	0.4	180	80	450	120	40	80	30	60	..	..
1518[2]	1.6	0.2	1.0	1.9	2.3	1.3	1.0	0.5	200	130	240	120	40	110	60	70	7	1
Clostridium sporogenes	...	...	0.3	...	1.5	...	0.4	...	470	...	171	...	14	...	8	..	..	..
Medium	1.5		0.1		0.5		0.2		117		15		24		85		148	

Notes:

1) Vegetative cells and spores, respectively

2) 1503 and 1518 are thermophiles (flat-sour types).

Table 9. Metal content of A. marinus and P. cuprodurans cells grown on basal seawater medium at 20 ± 2 C, harvested at stationary growth, and washed three times with 150-180 ml of cold 0.5 M ammonium formate. Concentration factors are calculated from the metal contents of the basal seawater medium (Jones, Royle and Murray, 1976).

Metal	Concentration in ppm/dry cell weight of cells Bacterial Cultures			
	A. marinus		P. cuprodurans	
	Concentration	Concentration Factor	Concentration	Concentration Factor
Na	5600	0.7	255	0.03
Mg	1580	1.6	292	0.3
Ca	790	2.6	272	0.9
K	700	2.3	36	0.1
Zn	122	622	112	571
Fe	257	1548	422	2542
Cu	14.4	720	19	950
Pb	9.7	510	3	158
Ni	1.7	106	4.5	281
Mn	1.7	131	0.9	69
Cd	2.8	1400	4.6	2300

Table 10. Trace metal and major ion concentrations of 23 bacteria grown on basal seawater medium (Jones, Royle, and Murray, 1976).

ORGANISM	Dry Weight	Concentrations of metals in ppm dry weight										
	g	Cu	Pb	Cd	Zn	Ni	Fe	Mn	Na	K	Mg	Ca
B-16 (N.C.M.B. 19)	0.281	22	3	3.9	159	1.5	262	0.4	299	36	420	267
Pseudomonas sp. (N.C.M.B. 130)	0.147	49	2	8.8	180	1.5	365	0.5	327	55	156	286
Pseudomonas cuprodurans	0.219	19	3	4.6	112	4.5	422	0.9	255	36	292	272
Vibrio marinus	0.135	29	2	10.4	101	1.5	351	1.5	357	89	359	165
U.N.H. 1	0.641	24	15	2.0	168	3.4	343	2.0	11	47	440	80
U.N.H. 3	0.470	33	15	4.3	206	4.7	290	0.4	192	85	536	216
U.N.H. 4	0.287	19	3	4.9	44	1.5	137	16	25800	4184	8939	N.D.
U.N.H. 5	0.172	50	45	6.4	217	3.5	314	0.3	2565	1166	705	186
U.N.H. 7	0.105	84	96	6.6	308	8.5	462	1.0	531	123	698	266
U.N.H. 11	0.739	10	1	1.4	57	0.7	132	0.3	433	202	391	70
U.N.H. 14	0.070	24	11	1.7	302	2.9	436	1.4	315	143	4822	830
U.N.H. 15	0.228	21	4	1.8	153	0.9	205	2.2	281	114	737	150
U.N.H. 16	0.086	16	27	3.5	118	1.7	245	1.2	232	46	672	104
U.N.H. 23	0.207	17	11	1.7	161	1.0	289	0.4	290	87	663	136
U.N.H. 25	0.316	8	4	2.6	53	1.5	147	0.5	392	57	1362	596
U.N.H. 26	0.613	22	20	3.4	169	0.8	222	0.7	227	326	463	160
Pseudomonas fluorescens	0.454	19	39	2.9	93	2.6	99	3.1	365	99	544	48
Escherichia coli	0.335	11	11	3.1	76	0.6	111	2.7	3341	1789	1351	42
Staphylo-coccus aureus	0.482	14	30	4.3	104	0.6	86	4.4	66	207	776	112
Pseudomonas ovalis	0.379	12	18	0.4	108	2.8	256	0.5	211	26	660	834

Table 10. Continued

ORGANISM	Dry Weight	Concentrations of metals in ppm dry weight										
	g	Cu	Pb	Cd	Zn	Ni	Fe	Mn	Na	K	Mg	Ca
Bacillus megatherium	0.143	36	13	1.0	186	2.4	339	10.5	298	149	1178	1655
Arthrobacter marinus												
(average)		14	10	2.8	122	1.7	257	1.7	5600	700	1440	790

Table 11. Weight as ppm dry weight of Zn, Fe, Cu, Pb, Ni, Mn, and Cd in cells of 5 cyanobacterial cultures, dried after growth in medium C at 34 C. Concentration factors (C.F.) indicate the increase of metals in cells compared to medium (Jones, Murray and Carr, 1977).

Cyanobacterial Culture	Zn		Fe		Cu		Pb		Ni		Mn		Cd	
	Cells	C.F.	Cells	C.F.	Cells	C.F.	Cells	C.F.	Cells	C.F.	Cells	C.F.	Cells	C.F.
Anabaena cylindrica	93	1257	6287	6287	38	633	14	700	7.2	720	137	228	1.5	75
Anabaena variablis	48	649	659	659	53	883	9	450	5.1	510	51	85	2.3	115
Anacystis nidulans	81	1095	1062	1062	76	1266	13	650	2.1	210	76	126	2.3	115
Chlorogloea fritschii	109	1473	1846	1846	61	1017	10	500	6.3	630	123	205	2.9	145
Nostoc muscorum	479	6473	8279	8279	159	2650	20	1000	9.4	940	1183	1972	9.1	455
Average Me in Cells	162		3626		77		13		6.0		97[1]		3.6	
Average C.F. in Cells		2189		3626		1290		660		602		161[1]		181
Me content in medium (Calculated) µg/liter	74		1000		60		20		10		600		20	

Notes:

1) The figure for N. muscorum not included

continuing cultivation on the altered medium long enough to produce a reasonably complete adaptation of the organism to its new environment, the experiments can be adequately controlled, and an observable change of properties and composition...can be obtained."

MATERIALS AND METHODS

Organisms

I used three bacterial cultures during this investigation. They were Escherichia coli Hfr C (UNH culture collection), Arthrobacter marinus (ATCC 25374), and Pseudomonas cuprodurans (UNH culture collection). Wirsen (1966) isolated A. marinus and Cobet (1968) classified it. Roche (1966) originally isolated P. cuprodurans and McCarthy (1971) identified the culture.

I employed the non-marine bacterium for phases of the investigation where I did not wash the cell material. I used A. marinus only during preliminary studies. P. cuprodurans which had less tendency to form flocs or aggregate on culture vessel walls was more convenient for continuous cultivation.

A. marinus and P. cuprodurans have been maintained at the Department of Microbiology of the University of New Hampshire.

Labware Preparation

I prepared reagents and growth media in borosilicate glassware, and stored reagents and acidified samples in polypropylene bottles. Teflon (polytetrafluoroethane, E.I. Dupont, Wilmington, Delaware) beakers and covers were used for acid digestions. Silica crucibles were used for dry ashing.

I cleaned labware by washing for two cycles in a Model 4000 automatic glass washer (Betterbuilt Machinery Corporation, Saddlebrook, New Jersey), and soaking in concentrated NHO_3 for at least 18 h. Next, I rinsed labware copiously with, soaked it overnight in, and re-rinsed it in double distilled deionized water (Milli-Q water polishing system, Millipore, Bedford, Massachusetts) (Q2W). Labware was dried in a laminar flow hood and wrapped in polyethylene film until use.

Media

Except for media used in the taxonomic study, I employed two growth media. I cultivated E. coli on Nutrient Broth (Difco, Detroit, Michigan), and cultivated A. marinus and P. cuprodurans on modified 2216E broth based on 2216E of Oppenheimer and ZoBell (1952) consisting of 0.1 % (w/v) Bacto-peptone (Difco) and 0.1 % (w/v) yeast extract (BBL, Cockeysville, Maryland) in synthetic seawater adjusted to 26 $\pm$ 0.5 ppt salinity.

Synthetic seawater for media in the preliminary phases of the study was that of Lyman and Fleming (1940). Subsequently, I adopted the similar synthetic seawater described by Kester et al. (1967).

Growth Measurements

I determined bacterial population densities routinely by observing absorbance at 420 nm, A_{420}, on a Zeiss PMQ II spectrophotometer (Carl Zeiss, Oberkochen, Weltenberg, Germany).

In order to relate these growth measurements to other techniques commonly used to determine bacterial concentrations, I prepared a series of eight two-fold dilutions in Lyman and Fleming's seawater, LFSW, from an 18-h P. cuprodurans broth. For each dilution, I determined turbidity, viable and direct count, cell mass (dry weight) and protein concentration.

I obtained viable counts by spread plating (Buck and Cleverdon, 1960) and observed direct counts using a Petroff-Hausser counter and a Zeiss WL standard research microscope with phase contrast optics (Carl Zeiss, Zena, Germany) at 400 x magnification. I determined dry weight by filtering 10 ml of a given dilution through a tared 25 mm, 0.45 μm pore size type HA Millipore filter (Millipore Corporation, Bedford, Mass.); drying at 100 C overnight, cooling in a desiccator over silica gel, and weighing on an analytical balance. I processed each dilution in triplicate.

To assay for protein, I used a modification of the Lowry (1951) protein assay. Reagents for this assay were:

A: 2 % (w/v) Na_2CO_3 in 0.1 N NaOH

B-1: 3.2 % (w/v) $CuSO_4$ (anhydrous) in Q2W

B-2: 10 % (w/v) K-Na tartrate in Q2W

B: 1 volume B-2, 1 volume B-1, 8 volumes Q2W; discard after 24 h.

C: 50 volumes reagent A, 1 volume reagent B; discard after 24 h.

E: 0.5 N Folin-Ciocalteau reagent, diluted from 2 N reagent stock with reagent A (Fischer, Fair Lawn, New Jersey).

As a standard, bovine fraction V (Difco) was diluted with Q2W to provide protein concentrations from 20-100 µg/ml. To harvest, I centrifuged cells in a given dilution at $1.8 \times 10^4 \times g$ for 10 min, and resuspended the pellet in 2.0 ml reagent A. After 30 min, I transferred 1.0 ml of sample to an assay tube and subsequently added 4.0 ml reagent C. Ten min later, I added 1.0 ml of reagent E to the mixture while agitating it in a Vortex mixer. After the color developed for 30 min, I determined A_{750} using 2 cm pathlength cuvettes. I calculated protein concentrations of unknowns from the regression equation describing the standard curve.

Maintenance and Preservation of Cultures

Prior to 1973, P. cuprodurans and A. marinus were maintained on modified 2216E agar slants. Subsequently, 48 h cultures grown in modified 2216E have been brought to 20 % (w/v) dimethyl sulfoxide (DMSO) (Eastman, Rochester, New York) and stored at - 90 C. E. coli Hfr C, a UNH Department of Microbiology stock culture, was stored in 20 % (w/v) DMSO. I prepared working slants of each of the marine bacterial cultures by inoculating 2.0 ml modified 2216E broth with a loopful of cells from a thawed DMSO suspension. After 24 h incubation at 25 C, I prepared a streak plate and inoculated a second sterile broth from the first broth. I examined the streak plate and first broth for evidence of contamination.

If there was no sign of contamination, I prepared modified 2216E working slants. After 48 h incubation at 25 C, I transferred slants to 4 C for storage. I prepared working slants at intervals not exceeding three months.

Taxonomy of P. cuprodurans

Stains

Stains used for identification of the culture included Hucker's modification of the gram stain, Bartholomew and Mittwer's "cold" spore stain, Liefson's flagellum stain, Hiss' capsule stain, Burdon's fat droplet stain, and acid fast stain (all from Manual of Microbiological Methods, 1957). I observed the stained preparations and wet mounts through a Zeiss WL research microscope at 1250 x magnification. I observed the stained bacteria through the bright-field objective; wet mounts through the phase-contrast objective.

Electron Microscopy

Reagents: 0.1 M sodium cacodylate buffer, pH adjusted to 7.2 with 3 N HCl; (A) 0.25 M sucrose in buffer, (B) 0.1 M sucrose in buffer; (C) 0.25 M sucrose, 3 % (w/v) glutaraldehyde (Ladd, Burlington, Vermont) in buffer, 1 % (w/v) phosphotungstic acid (PTA) at pH 7.5

I fabricated a micro-perfusion chamber by cutting the closed end off a Beem capsule and punching a 4 mm hole in each of two end caps. The caps were used to retain a 0.45 µm pore size polycarbonate membrane (Nuclepore, Pleasanton, California) in position over each end of the

capsule. I filled the capsule with an aliquant from a 12 h culture of P. cuprodurans in modified 2216E broth. I then placed the perfusion chamber into a scintillation vial containing solution C, and stirred it for 12 h at room temperature. I replaced solution C with solution B for one h. I placed a droplet of the fixed cell preparation on a formvar-coated Cu grid for one min, then stained it with PTA, removing the excess stain with Whatman #1 filter paper (W & R Balston, Ltd) (Hayat, 1972). After the grids were air dried, I stored them in a desiccator until I observed them with a Philips EM-200 transmission electron microscope (TEM).

Cultural Characteristics

Using the criteria recommended in the Manual of Microbiological Methods (1957), I evaluated the colony morphology and broth culture characteristics of P. cuprodurans on the following prepared media: extract agar (Difco), potato-dextrose agar (BBL) and modified 2216E broth. All media were prepared in LFSW at 26 ppt salinity (75 % LFSW).

Nutrition and Physiology

Prepared media employed for nutritional and physiological tests included: Pseudomonas agars F and P (Difco), lead acetate agar (Difco), Simmon's citrate medium (BBL), Koser's citrate broth (Difco), Bacto-urea broth (Difco), MR-VP medium (BBL). These differential media were prepared in 75 % LFSW. Additional differential media used are listed below.

Nicotine agar (Squros, 1955): Nicotine (Eastman) 0.4 % (w/v), KH_2PO_4, 0.2 % (w/v), KCl 0.5 % (w/v), $MgSO_4$ 0.0025 % (w/v), $FeSO_4$ 0.0025 % (w/v), yeast extract (Difco) 0.01 % (w/v), Bacto-agar (Difco) 1.5 % (w/v) in Q2W and 75 % LFSW.

Gelatin stabs: gelatin (Difco) 12 % (w/v), extract broth (BBL) 9 % (w/v) in 75 % LFSW.

Indole production and nitrate reduction: KNO_3 0.1 % (w/v), trypticase soy broth 3.0 % (w/v) in 75 % LFSW. Inoculated tubes were incubated at 20 C. After growth, I assayed indole formation using Kovac's reagent, and determined nitrate reduction with the Greiss-Ilosvay reagents.

Hydrogen sulfide production: extract agar supplemented with cysteine 0.1 % (w/v) and $FeSO_4 \cdot 7\ H_2O$ 0.02 % (w/v) in 75 % LFSW.

Creatine utilization (Dubos and Miller, 1937): as sole nitrogen and carbon source: creatine 0.2 % (w/v), KH_2PO_4 0.005 % (w/v) in Q2W or 75 % LFSW; as sole carbon source supplemented above with $(NH_4)_2SO_4$ 0.05 % (w/v).

Ammonium or nitrate as sole nitrogen source: NH_4Cl or KNO_3 0.1 % (w/v), glucose 1.0 % (w/v), KH_2PO_4 0.005 % (w/v).

Carbohydrate utilization: Bacto-phenol red broth (Difco), or synthetic base: NH_4NO_3 0.1 % (w/v), K_2HPO_4 0.05 % (w/v), 1.0 ml bromthymol blue solution in 75 % LFSW. Bromthymol blue solution: dibromthymol sulfonphthalein 1.6 % in Q2W. I prepared the following carbohydrates as 5 % (w/v) stocks in 75 % LFSW and autoclaved them separately from the

base solutions: arabinose, dextrin, fructose, galactose, glucose, inosotol, lactose, maltose, mannitol, mannose, rhamnose, ribose, salacin, sorbitol, sucrose, thiotine and xylose. After autoclaving, I added 1.0 ml carbohydrate to 4.0 ml base in 18 x 180 mm culture tubes containing inverted Durham tubes. I inoculated broths then incubated them aerobically and anaerobically at 20 C.

Cellulose utilization: Whatman #2 filter paper soaked in solution of NH_4NO_3 0.1 % (w/v), K_2HPO_4 0.002 % (w/v), asparagine 20 µg/ml, glucose 1.0 % (w/v) in 75 % LFSW.

Ashby's nitrogen free medium: Mannitol 1.5 % (w/v), $MgSO_4 \cdot 6H_20$ 0.02 % (w/v), NaCl 0.01 % (w/v), $CaSO_4 \cdot 2H_20$ 0.01 % (w/v), Noble Agar (Difco) 1.5 % (w/v) in Q2W.

Thiotone broth (Blankenship and Doetsch, 1961): thiotone (BBL) 0.1 % (w/v), glucose 0.25 % (w/v), K_2HPO_4 0.25 % (w/v) in Q2W and 75 % LFSW.

Acetate, succinate and lactate utilization: sodium salt of citrate, succinate or lactate 1.0 % (w/v), NH_4NO_3 0.1 % (w/v), arginine 20 µg/ml, Bacto-agar 1.5 % (w/v) in 75 % LFSW.

Additional determinitive tests: starch hydrolysis (Pelzcar, 1965) by flooding colonies on starch agar with iodine solution; oxidase activity (Skerman, 1967) by spotting colonies with 1.0 % (w/v) tetramethyl-p-phenylenediamine hydrochloride solution; catalase activity by spotting colonies with 3 % hydrogen peroxide (Pelzcar, 1965); acetylmethyl-carbinol production by the method of Barritt (1936). I used the criteria presented in the Manual of Microbiological Methods

(1957) and Skerman (1967) for interpreting results.

I used the auxanographic technique of Zubrzycki, Levinson and Braverman (1969) to determine nutritional requirements. I assayed requirements for the following amino acids, nucleic acids and vitamins: leucine, isoleucine, phenylalanine, aspartate, glutamate, tryptophan, arginine, methionine, valine, histidine, lysine, serine, glycine, threonine, glutamine, adenine, guanine, uracil, thymine, thiamine, nicotinic acid, riboflavin, Ca-pantothenate, biotin, p-amino benzoic acid, pyridoxal, and folic acid.

Antibiotic Sensitivity

I used commercially prepared antibiotic discs to determine the sensitivity of P. cuprodurans to the following antibiotics: Ampicillin (Difco), Aureomycin (chlortetracycline, BBL), Carbenicillin (Difco), dimethylchlortetracycline (BBL), cephalothin (Difco), Erthromycin (Difco), Gantrisin (BBL), Gentamycin (Schering), Kanamycin (Difco), Penicillin G (Difco), Polymyxin B (Difco), Streptomycin (Difco), Terramycin (oxytetracycline, BBL), Tetracycline (Difco). I placed the discs on 2216E Marine Agar (Difco) plates which had been spread with 0.1 ml of a 1 x 10^9 cell/ml P. cuprodurans suspension and incubated the plates at 25 C for 48 h.

DNA Extraction and Mole % (G + C) Determination

I extracted deoxyribonucleic acid (DNA) by Marmur's (1961) technique, and quantified yields by the diphenylamine reaction (Burton, 1956). I determined mole % (G + C) from

the melting curve of P. cuprodurans DNA in 0.01 M sodium phosphate, 0.001 M ethylenediamine tetracetic acid (EDTA) buffer at pH 7.2 (Marmur and Doty, 1962; Owen, Hill and LaPage, 1969).

Ionic Requirements

I determined specific ionic requirements by preparing sterile 10 x stocks of each component of a medium containing NH_4Cl, 0.1 % (w/v), glucose 1.0 % (w/v), K_2HPO_4 0.005 % (w/v), Lyman and Fleming's artificial seawater at 26.5 ppt salinity. I prepared the final medium by deleting one or more of the salts from Lyman and Fleming's artificial seawater. I considered ions non-essential if the inoculated medium was turbid after 48 h at 20 C.

Effect of Temperature on Growth Rate

I determined the relationship between temperature and μ', the specific growth rate, by growing P. cuprodurans in 100 ml modified 2216E at 200 rpm at 7, 15, 20, 25, 30, 35, 40, and 45 C in a Metabolyte water bath shaker (New Brunswick Scientific, New Brunswick, N.J.). I ran growth curves in duplicate, and inoculated cultures with a standard inoculum. For consistency, the inoculum used for all experiments was the equivalent of 1 ml from a 12 h starter culture whose $A_{420nm} = 1.0$.

Effects of Autoclaving Growth Medium

pH Changes

I measured pH with a Corning pH meter, Model 12

(Corning Glass Works, Corning Glass Works, Corning, N.Y.) and a Polymark prefilled combination electrode (Markson Scientific, Del Mar, California). I measured pH of modified 2216E at room temperature before and after autoclaving the medium.

Spectral Differences Between Autoclaved and Filter-Sterilized Medium

I divided a 100-ml batch of modified 2216E into two aliquots, autoclaving one and filter-sterilizing the other, and performed two spectral scans on a Beckman Model DB-G grating spectrophotometer (Fullerton, California). On the first scan, I used the filter-sterilized medium as a blank. On the second scan, I used autoclaved medium as a blank.

Electrochemical Analysis

I sterilized aliquots of a batch of modified 2216E in ASW in 26.5 ppt either by filtration or autoclaving at 121 C for 15 min. I adjusted the pH of the autoclaved sample to that of the filtered medium, with 0.1 N HCl, then analyzed both preparations by differential pulse polarography on a mercury dropping electrode. Mr. Leo J. Spencer performed the analyses using a Model 174 A polarographic analyzer with a Model 315 automated electroanalysis controller (Princeton Applied Research, Princeton, N.J.) He scanned the samples at a two drop/sec drop-rate over an electropotential range from 0.0 to -1.5 V at a one mV/sec scan rate.

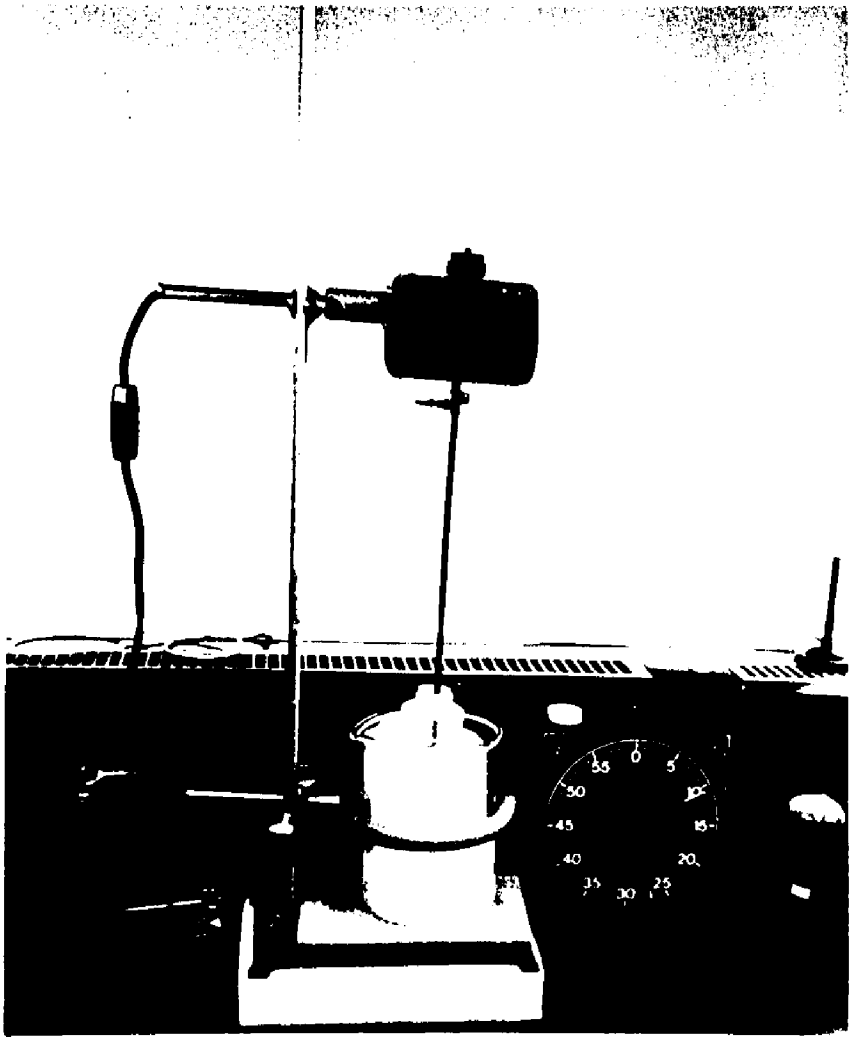
Scanning Electron Microscopy (SEM)

Nuclepore membranes through which I had filtered samples were mounted and coated with a 75-100 Å thick layer of carbon. I observed the membranes with a Model 1000 A scanning electron microscope (Applied Metals Research, Bedford, Massachusetts) at either 10 or 20 kV, 39° T, and 830 to 4,590 x magnification, and had residues on the membrane analyzed for elemental composition using an EDAX Model 711 energy dispersive analyzer (Prairie View, Illinois). Ms. Eleanor Tveter Gallagher performed the SEM and EDAX analyses.

Wash Procedure

I harvested bacterial cells by centrifugation at 1.8×10^4 x g for 10 min. After discarding the supernate, I resuspended the pellet in 20 ml chilled (4 C) Q2W. If bacteria were harvested in more than one centrifuge bottle, I pooled the pellets during the initial resuspension procedure. Once the pellets were pooled, I added an additional 80 ml chilled Q2W and vibromixed the sample on ice for 10 min. The assembled vibromixing apparatus (Vibromixer, Chemapec, Inc., Hoboken, N.J.) was shown in Fig. 1. I centrifuged and resuspended the sample a third time. Following the final wash, I centrifuged the sample once more for 10 min, discarded the supernate, then stored the polycarbonate bottle containing the pellet at - 90 C for several h. Freezing the pellet facilitated quantitative transfer to a tared teflon beaker by eliminating the need to wash the cell material from the centrifuge bottle.

Fig. 1. Vibromixer in operation. Ice in beaker kept sample chilled during agitation period. Dark-room timer shut off mixer at end of timed agitation period. Polystyrene block beneath ring stand dampened vibration and reduced noise. Polycarbonate centrifuge bottle was held in position by preformed pocket in ice.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Ash Procedure

Once I had transferred a sample to a teflon beaker, I dried it to a constant weight at 110 C and ground it to a fine powder in an agate mortar and pestle. I weighed a 150 to 200 mg sample onto a tared 25 mm Whatman #42 filter. I determined the sample weight to $\pm$ 10 µg keeping a static-master pulonium source and beaker of fresh silica gel in the weighing chamber during measurements. After weighing, I folded the filter with forceps to prevent sample loss, then placed it into a tared silica crucible.

I handled samples in batches of eight to 20 crucibles. I precharred each batch at 300 C, then treated each sample with 1.0 ml concentrated redistilled HNO_3. When the acid had evaporated to dryness, I returned the crucibles to the muffle furnace and ashed the samples at 450-500 C until the residue was light grey. This step required about 20 h. I cooled the samples over silica gel in a vacumm desiccator, then weighed them to determine ash yields.

I then redissolved ash residues and refluxed them in concentrated redistilled HNO_3 until only a light yellow, crystalline residue remained. I evaporated off the NHO_3 and redissolved the residue in 2.0 ml concentrated redistilled HCl.

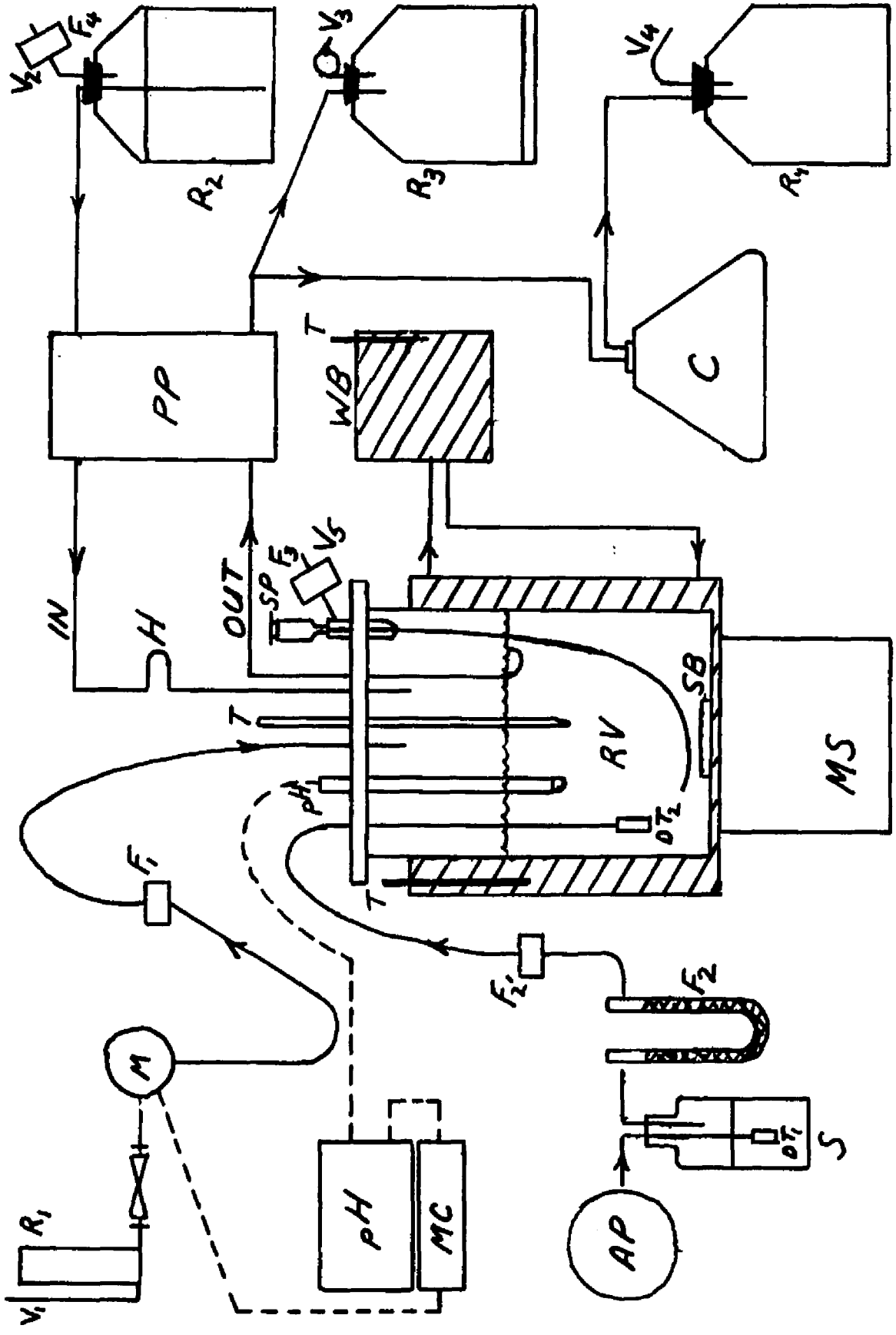
I filtered the dissolved sample through a Whatman #42 filter into a 25 ml volumetric flask and brought it to 25 ml with Q2W washings from the crucible. I also filtered the washings. I transferred each sample to a polypropylene vial and stored it at -4 C until analyzed.

I processed at least one filter paper control with each batch of samples.

Continuous Cultivation of P. cuprodurans

The apparatus assembled for continuous cultivation of P. cuprodurans was shown schematically in Fig. 2. The reaction vessel was a glass Brewer jar which had been graduated at 100 ml intervals to two liters with a diamond tipped pen. The reaction vessel head, manufactured by Diaphragm Industries (Portsmouth, N.H.), was a reenforced 12 mm ethyl propylene gasket with holes punched to accept the continuous cultivation apparatus fittings. The Physics Department, University of New Hampshire, fabricated an aluminum head clamp. Continuous culture apparatus fittings passing through the head included a gas dispersion tube, combination pH electrode, titrant inlet tube, glass thermometer, medium inlet and outlet tubes, and a sampling port. The sampling port consisted of a side-arm test tube from which I had removed the bottom. I fitted a Balston type 90 filter-housing with a type AA filter (Balston, Inc., Lexington, Massachusetts) onto the side arm to provide venting from the reaction vessel and covered the tube with a serum cap through which I had inserted a stainless steel canula. The canula was covered with 0.3 mm I.D. silicone tubing which extended into the growth medium. I used 5.0 ml disposable syringes to draw samples from the reaction vessel.

Fig. 2. Continuous cultivation apparatus, schematic diagram. Legend: AP, air pump; C, centrifuge; DT_1 and DT_2, gas dispersion tubes, F_1, glass fiber filter for influent 0.01 N NaOH; F_2, glass wool prefilter; F_2, 0.2 μm air inlet filter; F_3, air vent filter; F_4, air vent filter; H, heated section of medium inlet tubing; IN, medium inlet tubing; M, magnetic valve for NaOH influent line; MC, magnetic valve controller; MS, magnetic stirrer; OUT, medium outlet tubing; pH, pH meter and combination electrode; PP, peristaltic pump; R_1, titrant reservoir; R_2, 20 liter medium reservoir; R_3, preliminary effluent reservoir; R_4, 20 liter supernate reservoir; RV, reaction vessel; S, air sparging bottle; SB, stirring bar; SP, sampling port with 5 ml syringe; T, thermometer; V_{1-5}, vent tubes; WB, water bath-water jacket system; solid lines, tubing through which fluids are passing; broken lines, electrical connections.


V2
F4
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IN
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MS
pH
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T
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F2
M
R1
V1
pH
MC
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OT1
S

The reaction vessel was immersed in distilled water in a polypropylene tub. A copper coil, through which water from a Tamson water bath (Neslab Instruments, Portsmouth, N.H.) circulated continuously surrounded the reaction vessel in the tub. The tub and reaction vessel were supported by a magnetic stirrer which drove the teflon coated stirring bar within the reaction vessel.

I pumped water-saturated, filter-sterilized air through a gas dispersion tube into the reaction vessel from a Silent Giant aquarium pump. I monitored and controlled pH with a Model 28 pH meter and Model 11 titrator (Radiometer, Copenhagen), respectively. I metered 0.1 N NaOH from a one-liter volumetric flask (R_1) through a magnetic valve controlled by the titrator. The titrant passed through a type AP 15 Millipore filter just before entering the reaction vessel.

A Model 1201 peristaltic pump (Harvard Apparatus Company, Millis, Massachusetts) controlled medium inflow and discharge. Inlet tubing was 6.35 mm ID silicone, and outlet tubing was 7.87 mm ID silicone. To minimize inlet flow pulsation, I placed wyes in the tubing on either side of the pump and divided the inlet flow between the two pump heads. I also joined the pump outlet with the reaction vessel inlet tube via a one M section of 0.3 mm ID resistance tubing. The outflow line had two alternative flow paths . Tubing (7.87 mm ID) to a reservior (R_3) provided for disposal of effluent during non-steady state periods, and 0.3 mm ID tubing carried the effluent to the

centrifuge for harvesting.

A Szent-Gyorgi continuous flow apparatus in a Sorvall RC-2B centrifuge (Ivan Sorvall Inc.) continually hargested P. cuprodurans in the effluent distributing the flow among eight 50-ml stainless steel tubes rotating at $1.6 \times 10^4 \times g$. I collected supernate in a 20-liter glass carbouy (R_4).

I transferred modified 2216E in ASW adjusted to 26.5 ppt salinity to R_1 via the pressurized filtration system depicted in Fig. 3. An 18.9 liter pressure vessel (Gelman Instrument Corp., Ann Arbor, Mich.) was pressurized to 3.16 kg/cm^2 driving the medium through two 142 mm process filter holders (Millipore) stacked in series (Fig. 3) and into R_2 via reservoir's delivery tube. In first filter holder (H_1) were a 125 mm type AP 15 prefilter (Millipore); and a 142 mm 0.2 µm pore size filter (Nuclepore). The filters were separated by 125 mm dacron mesh (Millipore) and were not sterilized. The lower holder (H_2) was sterile and contained a 0.2 µm pore size filter. After the filtration process was completed, the system was bubble point tested at 4.57 kg/cm^2.

During operation, I monitored temperature, pH, A_{420}, flow rate, and reservoir (R_2) volume. To check the accuracy of the Radiometer pH meter, I determined the pH of samples drawn for A_{420} observations. I observed A_{420} on a PMQ II spectrophotometer generally diluting samples 1/10 in ASW adjusted to 26.5 ppt. After calibrating the inlet

Fig. 3. Pressurized filtration system (19.8 liter). Nitrogen from tank pressurized 18.9 liter vessel. Medium flowed from vessel into 20 liter glass reservoir via stacked 142 mm Teflon coated Millipore filter holders containing, in series, an AP 15 prefilter and 0.2 µm pore size filter in the first stage and a 0.2 µm filter in the second stage.

drop size, I determined flow rate by counting the number of drops in 30 sec, then multiplying by 0.08 to obtain ml/min.

I initiated an experiment by inoculating one liter of medium in the reaction vessel at pH 7.5 and 30 C with 4.0 ml of P. cuprodurans which had been resuspended from a 2216E agar slant (Cobet 1968).

After the culture reached the late log phase, I started the flow. Once the A_{420} stabilized (usually after 10 h), I harvested the effluent by continuous centrifugation.

At the end of an experiment, I flushed the reaction vessel effluent line with approximately 400 ml Q2W to insure that all of the expended medium had passed through the centrifuge and had been displaced by Q2W. As soon as the centrifuge rotor stopped, I decanted the supernate from the centrifuge tubes and set the tubes in ice. I pooled the eight pellets in a chilled 250 ml polycarbonate centrifuge bottle. First, I transferred the bulk of each pellet with a teflon policeman, then I added a small portion of Q2W to the tube and vibromixed for one min. This procedure effected total resuspension of the remaining cell material in the tube and permitted quantitative transfer of the cells to the 250 ml bottle. I brought the pooled, harvested P. cuprodurans to 100 ml with chilled Q2W and washed the cells according to the standard procedure (Materials and Methods--Wash Procedure).

Major Protoplasmic Elements

I performed C, H, N analysis in duplicate on a

Hewlett-Packard, F & M Model 180 CHN analyzer. I placed a 3-5 mg sample in an aluminum boat and weighed it on a Cahn analytical electrobalance with a direct input to the analyzer. I then covered the sample with Model 185 oxidizing catalyst (Hewlett-Packard) and analyzed it. I calibrated peak heights against National Bureau of Standard (NBS) acetanilide. Galbraith Laboratories, Knoxville, Kentucky, determined S concentrations.

Phosphorous

I used a modification of Fiske and Subbarow's (1925) technique for phosphate analysis. I obtained a standard curve from dilutions of a 10 μ mole PO_4/ml standards. Analyzing ashed samples, in solution, diluted 1:100 with Q2W, I obtained phosphate concentrations near the middle of the standard curve. For analysis, I added 1.0 ml standard or sample to a 22 x 175 mm glass culture tube. To this, I added 1.0 ml 5.0 N H_2SO_4 and 1.0 ml 2.5 % ammonium molybdate reagent, and agitated the tube with a vortex mixer. Next, I added 0.1 ml reducing reagent and 6.9 ml Q2W and mixed the solution again. Covering the tube with parafilm, I allowed the reaction to proceed for 10 min, then observed A_{660nm} on the PMQ II spectrophotometer using 5 cm path length cuvettes. I determined phosphate levels in the samples from the regression equation defining the standard curve. I also analyzed a blank using 7.9 ml Q2W and a control using 1.0 ml HNO_3 and performed assays in duplicate.

Trace-protoplasmic Elements

I used a Varian Techtron Model AA-4 flame atomic absorption spectrophotometer (Varian Techtron Pty. Ltd., Springvale, Australia) to analyze samples for Na, K, Ca, Mg, Fe, Zn, Cu, and Ni. I prepared standard curves from appropriate dilutions of 1,000 ppm standard solutions (Fischer). I analyzed standards before and after unknowns and at least once during analysis of unknown to provide at least triplicate data for standard curve construction. I determined concentrations of elements in unknowns from the regression equation of the standard curve.

The spectrophotometer was equipped with a digital readout which averaged absorbance over a 10 sec period. Accordingly, I analyzed each sample only once for each element.

Statistical Treatment

I applied appropriate statistical tests to the data, carrying out computations using subprograms of the Statpack program (International Business Machines, 1970) or programs developed from statistical treatments discussed by Weiner (1971) and Zar (1974).

Statistics most frequently computed were mean ($\bar{x}$) and coefficient of variation. The latter term was defined as:

$$\left(\frac{s}{\bar{x}}\right) \quad x \quad 100$$

where s is the standard deviation. I designed a number of multi-factorial experiments. Weiner delineated computations for these designs (1971).

RESULTS

Preliminary Investigation Defining Experimental Sources of Variation

Effects of Growth Medium Salinity And Sterilization Technique on P. cuprodurans' C, H, N Content

I conducted this investigation in order to standardize a methodology for obtaining bacterial samples, transforming them into a form suitable for elemental analyses, and performing the analyses. Preparing a list of the stages of the process most susceptible to systematic experimental error, I defined seven sequential processes: growth medium preparation, bacterial cultivation, harvest, washing, ashing, final preparation for analysis, and analysis. Before developing a standard procedure, it was necessary to determine the nature and extent of each process' contribution to the variability of the analytical data. Preliminary experiments evaluated medium preparation and cellular washing techniques.

Investigating the effect of growth medium salinity on the C, H, N content of P. cuprodurans, I grew the culture in autoclaved modified 2216E in 500 ml batch portions in one-liter Erlenmeyer flasks. In each of these experiments, I incubated 10 flasks for 48 h at 15 C at 200 rpm in a Psychrotherm incubator (New Brunswick). Half of the flasks contained modified 2216E at 12.8 ppt salinity, and the balance contained growth medium at 34.5 ppt salinity,

representing the salinity limits of the range over which P. cuprodurans was not restricted by salinity. I harvested the culture broths by centrifugation at $1.6 \times 10^4 \times g$, and pooled the pellets to form two subsamples. I washed the bacteria once in redistilled water, then dried them at 110 C to constant weight. I repeated the experiment three times, analyzing samples for C, H, N content. I did not perform trace element analyses on these samples.

The data, in Table 12, indicated that differences in the growth medium salinity did not affect P. cuprodurans' C, H, N content. The large variation among replications was primarily due to non-homogeniety of subsamples. To obtain uniform combustion in the analyzer, I should have ground the samples into a fine powder. In the preliminary experiment, I had removed the bulk of the dried bacterial mass, leaving only scrapings of the residual material for C, H, N analyses. Selecting the 0.80 confidence level minimized the probability of making a type two error, meaning accepting the null hypothesis when it should have been rejected.

I designed an analagous experiment to determine the effect of sterilization technique on the C, H, N content of P. cuprodurans. Dividing a batch of modified 2216E broth medium of salinity 34.5 ppt into two aliquots, I distributed one aliquot equally among five one-liter Erlenmeyer flasks and autoclaved the flasks. I distributed the remaining aliquot evenly by filter sterilization among five sterile one-liter Erlenmeyer flasks. The rest of the procedure was identical to experiments for determination of the effect of salinity on

Table 12. Effect of growth medium salinity of C, H, N content of P. cuprodurans.

	Element					
	C		H		N	
Salinity[1]	12.8	34.5	12.8	34.5	12.8	34.5
Replication						
1	38.69[2]	39.53	5.95	6.01	11.19	11.58
2	38.28	24.24	6.52	3.49	11.22	6.70
3	36.86	42.33	6.28	6.22	10.40	11.33
$\bar{x}_i$	37.94	35.37	6.25	5.24	10.94	9.89
Σdi	2.58		1.01		1.05	
$S_{\bar{d}}$	5.88		1.01		1.71	
$\lvert t \rvert_{obs}$	0.438		0.999		0.611	

$$T_{2[.80]} = 1.06$$

Notes:

1) Salinities are in ppt as LFSW in modified 2216E

2) C, N, N data are % dry weight

C, H, N content of bacterial cells.

There was a significant difference between P. cuprodurans harvested from filter sterilized modified 2216E and P. cuprodurans harvested from autoclaved modified 2216E (Table 13). Percent dry weight C, H, and N were all higher for P. cuprodurans grown in the autoclaved growth medium. I concluded that standardization of the growth medium preparation technique was important for minimizing variation in the elemental composition of bacteria.

Chemical Effects of Autoclaving Modified 2216E Medium

During the first month of this investigation, I had adjusted the pH of modified 2216E medium to 7.5 with NaOH prior to autoclaving. I observed a substantial precipitate which formed during autoclaving. To grow A. marinus and P. cuprodurans at pH 7.5 - 8.0 and to eliminate gross precipitation, I ceased preadjusting pH before autoclaving as suggested by Cobet (1968). As can be seen from inspection of Table 14, pH increased from 6.59 $\pm$ 0.168 to 7.85 $\pm$ 0.225 during autoclaving. This phenomenon instigated my investigation of other ramifications of autoclaving the complex medium.

Electochemical analyses using dropping mercury electrode differential pulse polarography suggested that several subtle changes occured in modified 2216E as a result of autoclaving. The data, which was only qualitative, indicated the Cu, Pb, and Ni were affected by autoclaving, but Cd, and Zn were not (Table 15). Cu and Ni underwent speciation changes which

Table 13. Effect of growth medium sterilization technique on C, H, N content of P. cuprodurans.

Sterilization Technique Replication	Element C		H		N	
	[1]F	[2]A	F	A	F	A
1	[3]39.53	44.27	6.01	6.16	11.58	12.23
2	24.24	48.40	3.49	6.36	6.76	13.19
3	42.33	46.34	6.72	6.26	11.33	12.71
$\bar{x}_i$	35.37	46.34	5.24	6.26	9.89	12.71
Σ_{di}	10.97		1.02		2.82	
$S_{\bar{d}}$	6.59		0.926		1.82	
$\lvert t \rvert_{obs}$	1.66		1.10		1.55	

$T_{2[.80]} = 1.06$

Notes:

1) Filtration

2) Autoclaving

3) C, H, N data are % dry weight.

Table 14. Effect of Autoclaving modified 2216E; pH Changes.

	Before Autoclaving	After Autoclaving	Δ pH
	6.29	7.51	1.22
	6.29	7.43	0.76
	6.67	7.96	1.29
	6.70	7.84	1.14
	6.63	7.75	1.12
	6.77	7.85	1.08
	6.66	8.05	1.39
	6.70	8.09	1.39
	6.65	7.95	1.30
	6.55	8.03	1.48
$\bar{x}$	6.59	7.85	1.22
s	0.168	0.225	0.207
CV_x%	2.55	2.86	17.0

Table 15. Effect of autoclaving modified 2216E; electrochemical changes.

[1]Peak Potential (V)	Element	[2]Peak Heights (μA)		Peak Height Ratios FS / Auto.
		Filter Sterilized	Autoclaved	
-0.14	Cu	0.073	0.042	1.74
-0.34	Pb	0.036	0.050	0.72
-0.53	Cd	0.330	0.340	0.97
-1.13	Zn	0.060	0.052	1.15
-1.26	Ni	0.030	0.010	3.33

Notes:

1) Peak potential is electropotential (volts) at which maximum occurs.

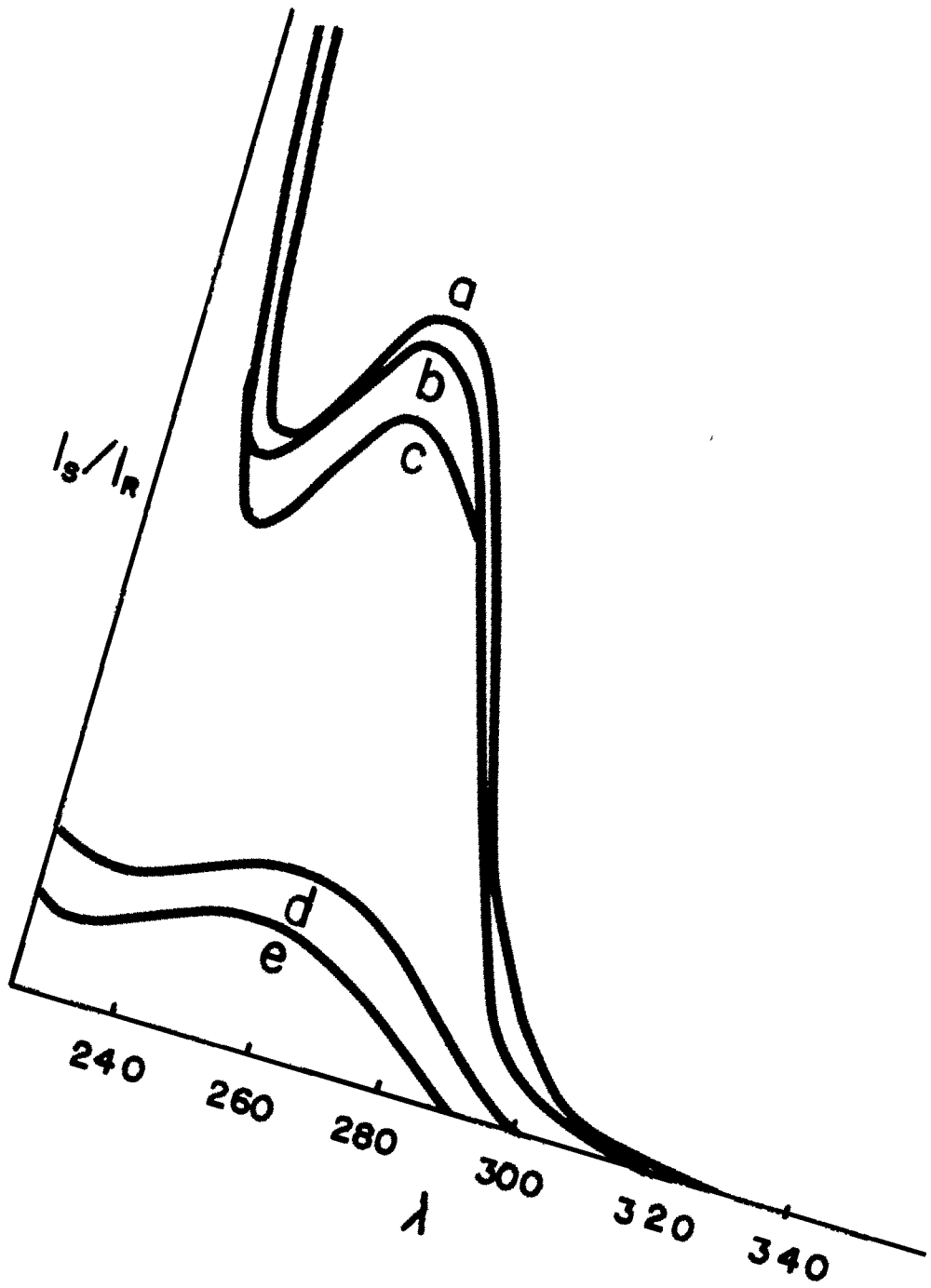
2) Peak heights are measured as vertical distance from maximum to a tangent from the minimums preceeding the peak.

decreased their respective peak intensities. While the Cu peak became smaller, the Ni peak became more broad suggesting that after autoclaving Ni was associated with a greater variety of complexes. The Pb peak became sharper after autoclaving. This may have reflected establishment of an equilibrium state between Pb complexes in their most stable states.

Since autoclaving caused a substantial pH change and discernible electrochemical changes, I speculated that qualitative changes in the organic fraction of the medium might also have occurred. To investigate this possibility, I compared UV spectra of filter-sterilized and autoclaved modified 2216E broth medium. Fig. 4 shows representative spectra of filter-sterilized medium and autoclaved medium compared with distilled water (curves a and c, respectively). After obtaining the spectrum for autoclaved medium, I centrifuged the autoclaved medium for 30 min at $3.9 \times 10^4 \times g$, and obtained a UV spectrum of the supernate (Fig. 4b). Comparision of autoclaved modified 2216E and centrifuged-autoclaved modified 2216E with filter-sterilized modified 2216E broth medium produced spectra d and e, respectively.

Although A_{260}/A_{280} did not vary among the samples, autoclaved modified 2216E medium absorbed light more strongly throughout the spectrum. Centrifugation caused absorbance diminution to a lesser extent than filtration did. I interpreted this to be a reflection of the relative efficiences of the two separation techniques and concluded that filtration removed undissolved organic matter from the growth medium and

Fig. 4. Effect of autoclaving on UV spectrum of modified 2216E medium. Analysis of uninoculated medium. a) autoclaved medium; b) autoclaved medium which had been clarified by centrifugation; c) filter-sterilized medium. Samples a, b, and c were compared against Q2W reference. Samples d and e same as a and b, respectively, except filter-sterilized modified 2216E broth medium was reference.


I_s/I_R
a
b
c
d
e
240
260
280
300
320
340
λ

that autoclaving did not cause any obvious qualitative changes in the organic fraction of modified 2216E broth medium.

Autoclaved Precipitate as Detected by Scanning Electron Microscopy

While preparing P. cuprodurans for TEM, I observed that when the culture was grown in autoclaved medium, the microscope grids were covered with detritus. This detritus was virtually absent from grids prepared with P. cuprodurans grown in filter-sterilized medium. Although no macro-precipitate appeared when modified 2216E broth medium was autoclaved, I could not discount the possibility that significant micro-precipitation occurred. To determine whether the peptone and yeast extract were completely dissolved before sterilization and if and what elements were associated with any precipitate, I conducted a simple experiment. Dividing a batch of modified 2216E into three 200 ml aliquots, filtering two of the aliquots through 0.2 μm Nuclepore membranes, autoclaving one of the filtered aliquots and the unfiltered aliquot, and filtering the two auotoclaved aliquots provided three membranes for SEM. Refiltering the filter-sterilized aliquot provided a fourth membrane for analysis. The four residues to be observed by SEM and energy emission x-ray spectrometry (EDAX) were: 1) initial filtration after preparing the growth medium, 2) refiltration of filter-sterilized medium, 3) filtration of autoclaved medium, and 4) filtration of autoclaved, filter-sterilized medium.

Figure 5a represented a low magnification micrograph of the membrane surface supporting residue 1. Performing EDAX, I was unable to coat the samples with Pt. Consequently, charging by organic matter in the sample caused a patterned shading effect of the micrograph. I observed numerous irregular particles ranging in size from 0.3 to 1.0 μm on the membrane after initial filtration.

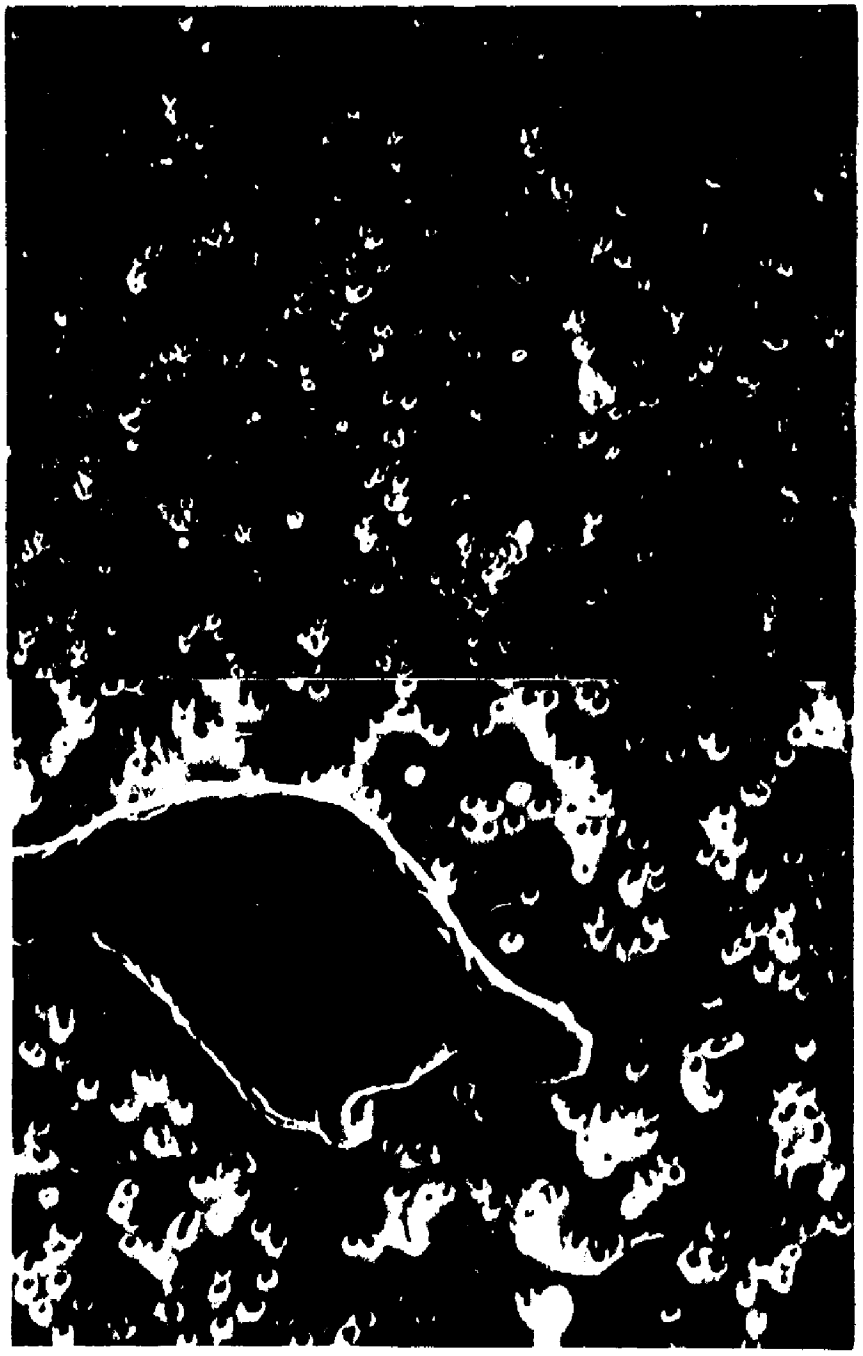
Although residue 2 was almost free from detritus (Fig. 5b), I observed a few large particles in the 4 x 10 μm size range, similar to the one shown in the micrograph. Having bubble point tested each membrane, I concluded that these particles could not have originated from the unfiltered modified 2216E. They were probably contaminants from either the first filtration reservoir flask or filtration apparatus reservoir. EDAX analysis revealed that the particle contained high concentrations of Si, Ca, Al, and Na, and significant concentrations of Mg, Cl, K, and Ti. I obtained zero background counts performing EDAX on the membrane itself.

Residue 3 (Fig. 5c) was a dense amorphous coating, consisting primarily of Ca, Mg, and P. I also observed high levels of Na, Si, Ti, and K, and lower concentrations of S, Cl and Ni. Residue 4 was not as dense as residue 3. Large crystalline aggregates, like the one shown in Fig. 5d predominated although amorphous aggregates were present.

I compared the composition of the crystalline residue with that of the amorphous residue by EDAX. Mg, P and Ca were major consituents of the non-crystalline fraction. The crystalline fraction contained Mg, P, and S,

Fig. 5. Scanning electron micrographs of filter residue of modified 2216E broth. a) Initial filtration, b) second filtration, c) filtration of autoclaved medium which had not been prefiltered, d) filtration of autoclaved medium which had been prefiltered. Magnification: a) 5,000 x, b) 10,000 x, c) 7,200 x, d) 850 x. All observed at 10 KV, 39° T.

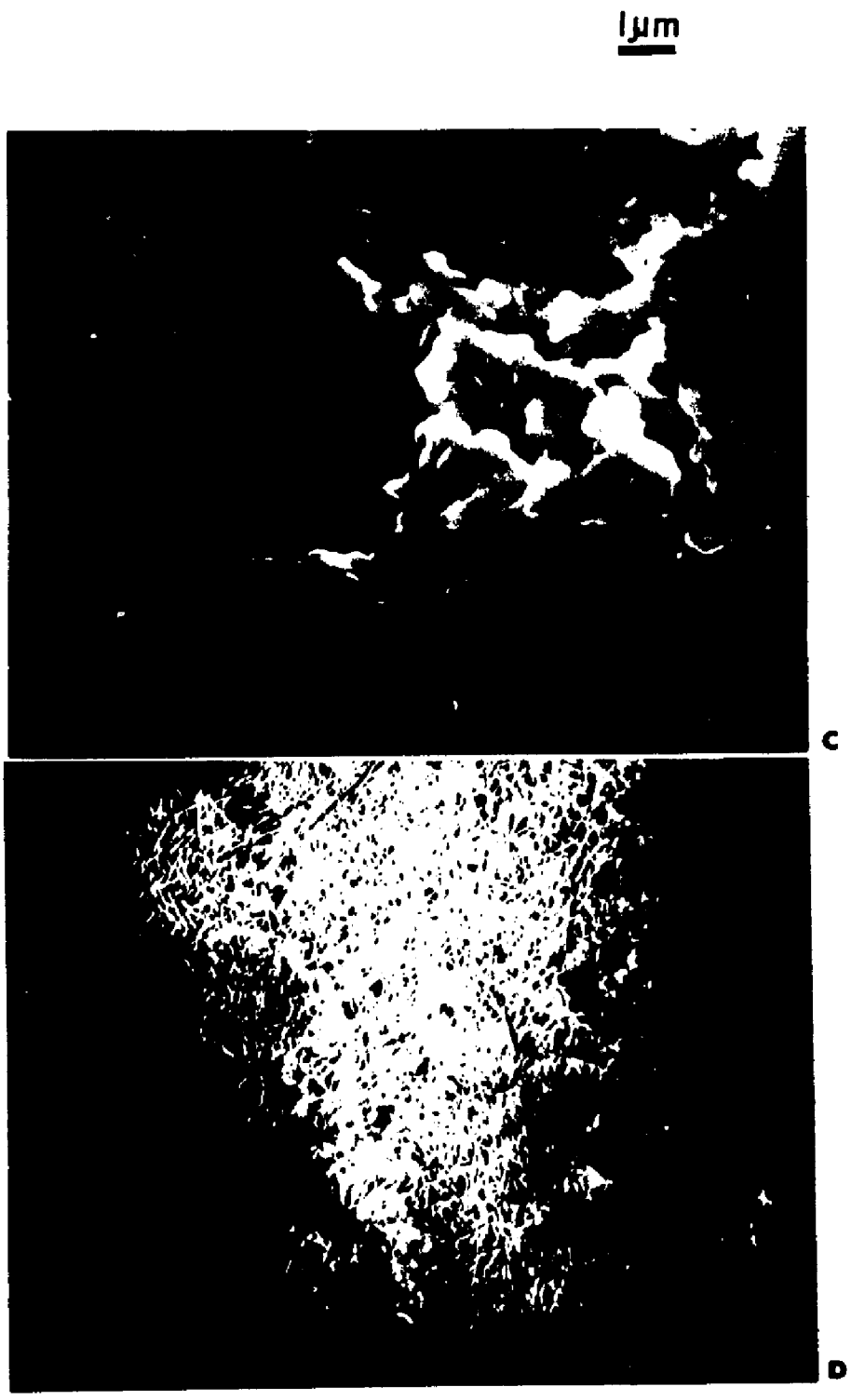
1 µm



A

B

1 µm


1µm
C
D
10µm

with lower amounts of Ca. An elemental map of S (Fig. 6a) and the 0.0 to 4.0 KeV EDAX spectrum of the non-crystalline matrix (Fig 6b, dots) superimposed over the same spectrum of the crystalline matrix (Fig. 6b, lines) suggested that S was associated with the crystalline matrix. The major Kα x-ray peaks were: Mg, 1.253 KeV; P, 2.013 KeV; S, 2.307 KeV; Ca, 3.739 KeV. Only the crystalline fraction contained S.

The SEM and EDAX data demonstrated that autoclaving had an effect of the chemistry of modified 2216E broth medium. Intending to perform elemental analyses on bacteria grown in the culture medium, I concluded that for this application filtration was preferred over autoclaving since the basic chemistry was less altered. The sterilization technique might even affect the growth of P. cuprodurans in culture.

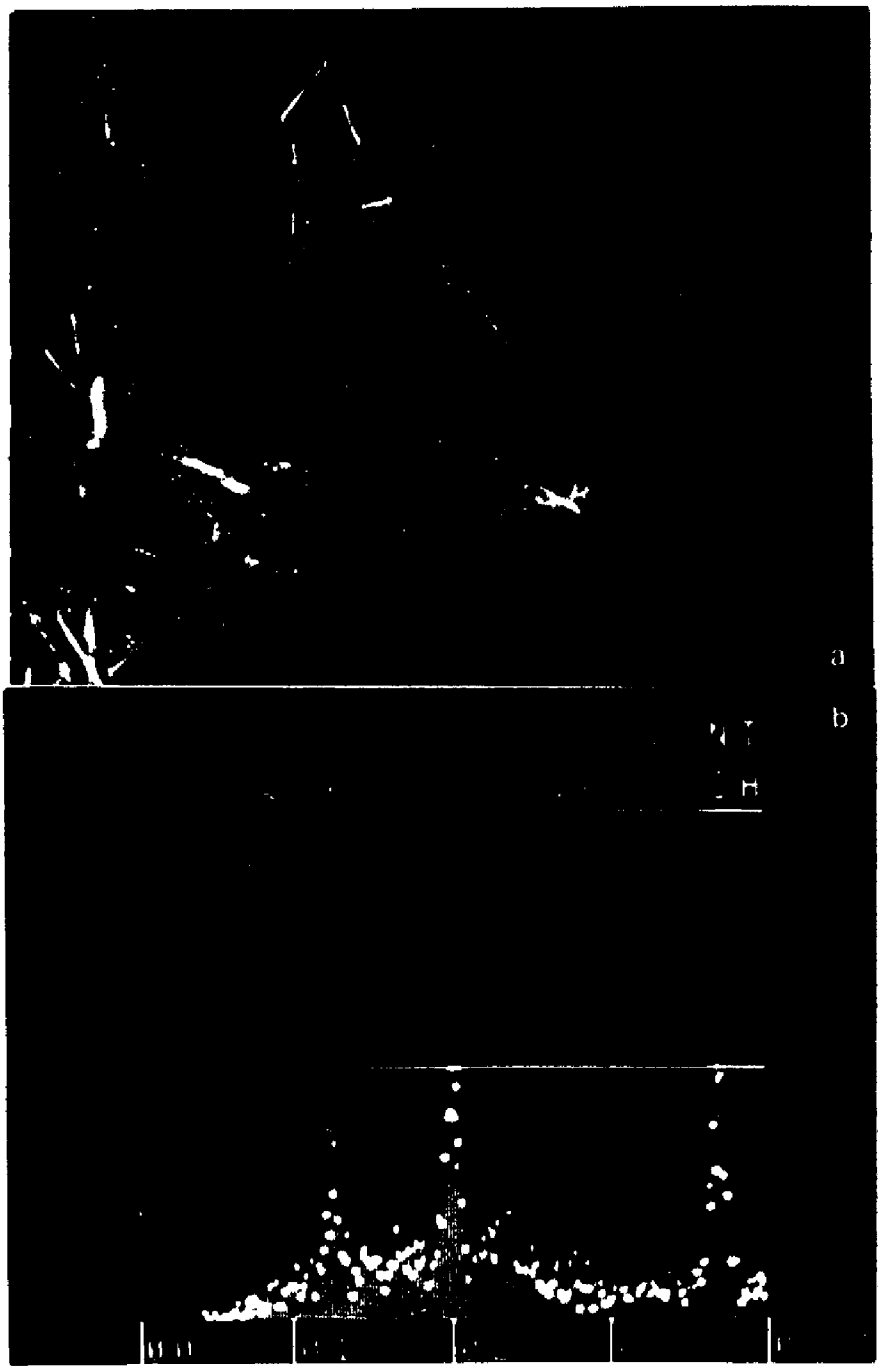
Effect of Autoclaving Modified 2216E Medium on Growth of P. cuprodurans

To test this hypothesis, I compared growth of P. cuprodurans in filter sterilized modified 2216E broth medium.

Dividing one liter of modified 2216E into two aliquots, I distributed the first 500 ml equally among five 250 ml sterile flasks, utilizing a Millipore 600 ml filling system equipped with a 0.22 μm pore size filter.

Before inoculating the medium, I adjusted the pH of each flask to 7.2--the pH of the preautoclaved medium. To

Fig. 5. a) Scanning electron micrograph of portion of residue from prefiltered, autoclaved modified 2216E broth medium, with sulfur map (dots). Magnification 7,300 x; 10 KV; 39° T. b) Energy dispersion spectra of crystalline (lines) and globular (dots) material on membrane.


1μm
a
b

adjust pH in the autoclaved aliquots, I titrated one aliquot with 0.1 N HCl, then added an identical volume of sterile titrant to each of the remaining flasks.

I inoculated three flasks of each type of sterilized medium with a standard inoculum of P. cuprodurans, then incubated them at 200 rpm on a Metabolyte waterbath shaker at 30 C, monitoring growth at A_{420nm} and pH as functions of incubation time.

I tabulated the results of the growth experiment in Table 16, observing no difference in either duration of lag or specific growth rate during logrithmic growth under these conditions. Sterilizaion technique did not effect the pattern of pH change (Table 17).

I concluded that the physico-chemical changes brought about by autoclaving did not affect bacterial growth. Autoclaving was a satisfactory method of sterilizing growth media in experiments where elemental composition data was not required. However, I still preferred filtration for defined media since it minimized post-sterilization manipulation of constituents which would normally be autoclaved separately then combined. Although physical chemical changes appeared during autoclaving of seawater media, bacterial growth was equivalent in autoclaved or filter sterilized media.

The Effect of Washing Harvested Bacteria

The presence of high concentrations of inorganic salts and organic constituents in the growth media indicated a

Table 16. Effect of sterilization technique on P. cuprodurans growth curve.

	STERILIZATION TECHNIQUE											
	Filtration Experiments						Autoclaving Experiments					
	(1)		(2)		(3)		(1)		(2)		(3)	
Time (h)	A_{420}	ΔlnA+10	A_{420}	ΔlnA+10	A_{420}	ΔlnA+10	A_{420}	ΔlnA+10	A_{420}	ΔlnA+10	A_{420}	ΔlnA+10
0	0.03	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.02	0.00
2	0.05	0.51	0.05	0.92	0.04	0.69	0.05	0.92	0.06	1.10	0.05	0.92
3	0.14	1.54	0.14	1.94	0.13	1.87	0.15	2.01	0.16	2.08	0.16	2.08
3.5	0.26	2.16	0.24	2.48	0.21	2.35	0.23	2.44	0.24	2.48	0.25	2.52
4	0.40	2.59	0.39	2.97	0.34	2.83	0.41	3.02	0.42	3.04	0.42	3.04
4.5	0.49	2.79	0.49	3.20	0.49	3.20	0.56	3.33	0.56	3.33	0.57	3.35
5	0.72	3.18	0.72	3.58	0.66	3.50	0.83	3.72	0.68	3.53	0.95	3.86
[1]μ		0.95		0.94		1.01		1.00		0.91		0.98
[2]r		0.990		0.994		0.996		0.992		0.997		0.995

Notes:

1) $\mu = \frac{\Delta(\ln A+10)}{dt}$, calculated as linear regression of Δ(lnA+10) on time (h).

2) r is regression coefficient for observed data on regression line.

Table 17. Effect of sterilization technique on pH changes during P. cuprodurans growth in modified 2216E medium.

	STERILIZATION TECHNIQUE					
	Filtration Run			Autoclaving Run		
Time (h)	1	2	3	1	2	3
0	7.21	7.20	7.18	7.23	7.14	7.01
2.0	7.28	7.29	7.23	7.19	7.11	7.12
3.0	7.15	7.21	7.20	7.08	7.01	7.02
3.5	7.03	7.09	7.04	7.00	6.96	6.94
4.0	7.01	7.02	7.02	6.96	6.87	6.09
4.5	6.89	6.85	6.87	6.88	6.82	6.90
5.0	6.91	6.89	6.87	6.88	6.82	6.87

requirement for a procedure for separating organisms from their contaminating menstruum. I hypothesized that the salinity of the growth medium affected the efficiency of any wash technique and designed a 2 x 4 x 3 factorial experiment to test the hypothesis. I grew and harvested P. cuprodurans as described for determining the effects of salinity on C, H, N content. During the harvest, I pooled the contents of nine flasks to form three subsamples. To test the effect of three wash reagents on the elemental composition of P. cuprodurans, I washed two sub-samples from each experiment with one of the reagents, and the third sub-sample with a second reagent, thereby reducing the possibility of incorrectly attributing compositional differences to the wash reagent when other sources of variation among experiments might have been responsible. I compared the effects of a single wash in redistilled water, 0.5 N ammonium formate (Riley and Roth, 1971), 0.01 N potassium phosphate buffer adjusted to pH 7.5 (Gomori, 1955), and no wash. I washed the cells by manually resuspending the pellet in the wash reagent, centrifuging at $1.6 \times 10^4 \times g$, and decanting the supernate. From eight experiments, I obtained three subsamples of each wash treatment at each salinity. I analyzed the samples for % C, H, N as fractions of dry weight (Table 18), and performed an analysis of variance on the data for each element (Table 19).

Exceptionally low C, H, N values for unwashed samples substantiated the requirement for a wash due to contaminating salts on unwashed cells. The ANOVA's demonstrated that

Table 18. Factorial analysis of effects of washing and growth medium salinity on % dry weight of *P. cuprodurans* C, H, N.

Element	Replication	[1]Wash: None		Deionized Water		0.5N Ammonium Formate		0.01 N Phosphate Buffer pH 7.5	
		[2]S=12.8	S=34.5	S=12.8	S=34.5	S=12.8	S=34.5	S=12.8	S=34.5
Carbon	1	30.91	16.18	38.69	39.53	37.78	47.70	36.84	39.16
	2	22.18	21.86	38.28	24.24	38.11	43.58	37.64	44.30
	3	23.94	20.73	36.86	42.33	34.36	44.93	36.00	43.47
Nitrogen	1	8.67	3.04	11.19	11.58	11.52	13.15	10.18	10.74
	2	6.26	6.40	11.22	6.76	11.40	13.53	10.54	12.74
	3	6.82	6.12	10.40	11.33	11.45	13.57	10.14	11.72
Hydrogen	1	5.30	4.96	5.95	6.01	6.58	6.68	5.60	5.61
	2	3.48	3.00	6.52	3.49	6.42	6.31	5.40	5.96
	3	4.06	3.68	6.28	6.22	5.89	6.31	5.18	6.03

Notes: 1) Cells harvested by centrifugation were resuspended and recentrifuged once in specified wash.

2) Salinity of growth medium was adjusted to either 12.8 ppt or 34.5 ppt.

Table 19. Summary of ANOVA for effects of washing and growth medium salinity on % dry weight *P. cuprodurans* C, H, N.

n=3, p=4, q=2

Element	Source of Variation	SS	df	MS	F	$F_{(0.95)}$ critical	Accept/Reject H_o
C	Wash	1281	3	427	27	3.24	Reject
	Salinity	11	1	11	0.7	4.49	Accept
	Wash x Salinity	248	3	83	5	3.24	Reject
	Error	250	16	16	---	---	
	Total	1790	23				
H	Wash	17	3	5.7	9.5	3.24	Reject
	Salinity	0.0	1	0.0	0.0	4.49	Accept
	Wash x Salinity	2	3	0.7	1.2	3.24	Accept
	Error	9	16	0.6	---	---	
	Total	28	23				
N	Wash	129	3	43	25	3.24	Reject
	Salinity	0.0	1	0.0	0.0	4.49	Accept
	Wash and Salinity	16.9	3	5.6	3.26	3.24	Reject
	Error	27.5	16	1.7	---	---	
	Total	173.4	23				

washing affected the % dry weight of all three elements. As in the earlier series of experiments, Table 12, salinity did not affect the C, H, N content of P. cuprodurans. However, salinity did affect the wash results (Table 19). The interaction was most pronounced for C. I obtained higher C yields in the 34.5 ppt salinity medium than in the less saline medium. The effect was less pronounced for N, and was not significant for H.

In a separate experiment, I grew A. marinus in autoclaved modified 2216E broth medium. I harvested and washed subsamples as I had done for P. cuprodurans in the previous experiment. After drying them, I transferred the samples to glass vials, and shipped them to Dr. Lindsay Murray at the University of Liverpool, England, for atomic absorption spectrophotometric analysis. She digested the samples in concentrated HNO_3 and analyzed for Na, K, Mg, Ca, Fe, Zn, Cu, Pb, Cd, Ni, and Mn. The data in Table 20 substantiated the requirement for washing. Na, Mg, and Ca were the most prevalent contaminants carried over from the medium. A single wash did not appreciably affect Fe, Zn, Cu, Pb, Cd, Ni or Mn concentrations. However, Na, K, Mg, and Ca were substantially reduced in washed cells. A. marinus lost more K and Mg in ammonium formate and phosphate buffer than in Q2W wash.

The data which I had accumulated to this point indicated that preparing the growth medium and washing the harvested bacteria were two processes which contributed sufficient variation to over-shadow the effects of other

Table 20. Effect of one washing on the elemental composition of A. marinus grown in autoclaved modified 2216E broth medium at 15 C.

Element	Wash			
	None	Q2W	0.5 N NH_4COOH	0.01 N K_xH_x PO_4 (pH 7.5)
Na	58,700 ppm	14,900	11,500	11,800
K	---	6,300	1,410	1,700
Mg	6,280	4,720	2,990	3,870
Ca	8,340	1,760	1,360	2,380
Fe	2,380	3,740	3,330	3,840
Zn	130	172	154	200
Cu	15	8	10	12
Pb	19	14	6	11
Cd	4	15	2	2
Ni	1	2	2	<1
Mn	3	2	2	2

sources of variation. These processes required refinement before I could investigate variation due to ash technique or final preparation of cells for analysis. I could, however, proceed to determine the variability introduced by storing and analyzing samples.

Sources of Variation After Samples Have Been Prepared for Analysis

There were a number of sources of variation associated directly with the analysis of the prepared samples. These included precision of repeated analyses of a given sample in rapid succession, after changing the operating parameters of the spectrophotometer, and after storing the sample for prolonged periods. I conducted a series of simple experiments to determine the contribution of each of these steps to the analytical variability.

The DI-30 module for the Varian AA-4 spectrophotometer was equipped with an adjustable averaging output which provided a digital output of average sample concentrations for a 10 sec interval. Under these conditions, replicate samplings of homogeneous samples should be identical. To test this, I assayed samples of ashed P. cuprodurans ten times, analyzing each sample for one of the following elements: Na, K, Ca, Mg, Fe, Zn, and Cu. The data (Table 21) demonstrated that the variation was over 4 % only for elements present below a ppm concentration. A major contributing factor to the increased variability for low concentrations was the amplification of photometer drift brought about by increasing the

Table 21. Precision of atomic absorption spectrophotometry for repeated analysis of P. cuprodurans as ash in acidified Q2W.

	Element						
	Na	K	Ca	Mg	Fe	Zn	Cu
[1]	14.06	16.78	14.83	15.57	1.29	0.246	0.130
	14.35	16.70	14.16	15.47	1.31	0.238	0.146
	14.16	16.86	15.02	15.67	1.28	0.255	0.136
	14.16	16.78	14.74	15.76	1.37	0.268	0.140
	14.35	16.86	14.93	15.57	1.32	0.265	0.126
	14.06	17.02	15.22	15.67	1.28	0.240	0.176
	14.25	17.18	14.45	15.57	1.33	0.244	0.129
	14.25	16.78	13.77	14.99	1.29	0.246	0.134
	14.35	17.02	15.02	15.47	1.30	0.260	0.136
	14.74	17.02	14.54	15.67	1.30	0.240	0.126
$\bar{X}$	14.74	16.90	14.67	15.54	1.31	0.250	0.138
s_x	0.198	0.152	0.445	0.214	0.028	0.0109	0.0147
$CV_x\%$	1.38	0.89	3.03	1.38	2.10	4.38	10.72

Note:

1) Figures represent µg element/ml sample.

instrument's sensitivity in order to detect the element. The solution was to increase the sample concentration within restrictions imposed by availability of sample.

In setting up the spectrophotometer to analyze for a given element, I had to optimize several parameters. The process was to a certain extent subjective. I positioned cathode lamps by hand along an optical rail to give maximal transmission of monochromatic light. I also adjusted the monochrometer wavelength filter and slit openings for optimal light transmission. The optical path-length and concentration of atoms in the light beam were functions of the position and intensity of the flame. Precision of analyses from one day to the next depended on the degree to which I replicated the alignment of the instrument.

To determine whether variations inherent in setting up the atomic absorption spectrophotometer would lead to significant differences in the data obtained, I analyzed ten samples for Na, K, Ca, Mg, Fe, Zn and Cu. After analyses for the seven elements was completed, I repeated the process and computed paired-t values for each element. The data in Table 22 was in µg element/ml sample and variation among samples was primarily due to differences in the dry weight concentrations of the sample, not experimental error. The comparison was strictly between the two values for each sample. At the 95 % confidence level, the differences between the two series of analyses were not significant for Na, Ca, Fe, Zn, or Cu, but were for K and Mg. These conclusions were a propos to the analyses performed to determine

Table 22. Effect of instrument alignment on analysis of P. cuprodurans ash in acidified Q2W.

Sample	Element							
	Na		K		Ca		Mg	
206	[1,2]14.99	[3]16.30	17.31	21.29	12.71	12.33	30.10	30.87
211	14.30	15.32	22.55	28.20	25.44	23.10	34.01	35.90
215	10.85	12.21	19.38	24.10	19.36	19.06	26.79	28.64
217	9.87	10.45	17.63	23.06	17.65	17.91	27.09	28.64
220	14.40	15.42	10.17	14.13	18.12	15.79	15.36	16.44
226	6.03	29.16	11.59	15.58	22.68	19.26	31.30	34.06
227	20.70	22.34	11.83	15.42	19.64	17.04	22.98	22.93
235	10.06	18.40	15.68	19.97	11.19	13.39	11.55	11.60
243	21.68	23.70	16.84	21.13	13.47	15.99	21.48	22.35
247	25.33	28.47	18.58	21.93	15.94	17.62	13.20	32.22
$\bar{x}$	14.82	18.40	15.68	19.97	17.62	17.14	25.19	26.36
$\lvert\Sigma d\rvert$	3.58		4.29		0.48		1.18	
$S_{\bar{d}}$	2.18		0.238		0.682		0.272	
[4]$\lvert t\rvert$	1.64		18.0		0.704		4.33	

Table 22. continued.

Sample	Element					
	Fe		Zn		Cu	
206	1.43	1.28	0.792	0.927	0.147	0.268
211	1.32	1.25	0.682	0.736	0.183	0.183
215	1.14	1.08	0.811	0.818	0.148	0.162
217	0.82	0.90	0.547	0.479	0.191	0.133
220	0.86	0.74	0.425	0.386	0.145	0.138
226	0.84	0.78	0.428	0.484	0.127	0.137
227	0.86	0.92	0.537	0.446	0.150	0.142
235	0.82	0.71	0.432	0.514	0.184	0.180
243	1.13	1.22	0.693	0.656	0.218	0.251
247	1.43	1.40	0.597	0.635	0.199	0.215
$\bar{x}$	1.06	1.03	0.594	0.608	0.169	0.181
$\lvert\Sigma d\rvert$	0.03		0.014		0.012	
$S_{\bar{d}}$	0.027		0.0227		0.0143	
$^4\lvert t\rvert$	1.36		0.603		0.818	

Notes:

1) Figures represent µg element/ml sample
2) Left column represents data from first instrument alignment.
3) Right column represents data from second instrument alignment.
4) $\lvert t\rvert_{\{10\}.95} = 1.83$

the effect of storage on the elemental composition of the sample since instrument alignment could account for observed concentration changes.

I stored ten of the samples prepared during the experiment to determine the effects of experiment, ash treatment, and organism (to be presented below) at 4 C for 11 months, then reanalyzed them. According to the data in Table 23, Na, K and Mg concentrations changed significantly during the storage period. Na differences were less between the two years than between the two days (Table 22), but the former $s_{\bar{d}}$ was also lower than $s_{\bar{d}}$ for the latter. This indicated that the change in Na concentration was analytical. Following a similar argument, I attributed the apparent change in Mg concentration to instrument alignment rather than leeching from the container.

The K concentration changes were even more substantial. Conducting an analagous experiment with P. cuprodurans which was analyzed in February 1975 and 1976, I observed a significant change in K concentration (Table 24). In this instance K increased rather than decreased. Coupling this data with the observation that K concentrations for a set of samples analyzed on two separate days demonstrated a more significant difference than the differences for any of the other elements assayed, I concluded that the difference could be attributed to instrument alignment.

During the earlier storage effect experiment, Dr. C.L. Grant suggested that the apparent change might have been due to loss of K from the Fisher 1000 ppm KCl standard.

Table 23. Effect of storage on elemental composition of E. coli ash in acidified Q2W.

Sample	Element							
	Na		K		Ca		Mg	
14	[1,2]22.0	[3]22.0	59.0	50.9	5.50	8.63	10.4	10.7
17	16.0	15.7	32.0	34.6	4.19	4.64	7.64	8.51
18	26.5	23.3	85.0	63.4	8.02	8.72	12.3	12.6
23	50.7	50.0	206.0	42.1	44.7	43.6	11.3	11.5
24	67.9	64.8	253.0	161.0	26.7	22.2	27.3	28.5
26	14.3	13.0	164.0	55.2	5.66	7.87	12.3	12.3
27	14.6	14.2	183.0	56.3	5.81	6.49	12.2	12.8
30	13.8	13.5	171.0	52.8	5.42	5.88	11.9	12.4
33	13.7	12.5	81.1	51.8	5.67	6.73	10.8	11.7
34	11.8	10.8	61.9	43.3	6.37	5.50	10.6	11.0
$\bar{x}$	25.1	24.0	129	61.2	11.8	12.0	12.6	13.2
$\lvert\Sigma d\rvert$	1.2		57.0		0.2		0.6	
$s_{\bar{d}}$	0.36		25.3		0.654		0.122	
[4]$\lvert t\rvert$	3.20		2.25		0.350		4.50	

Table 23. continued.

Sample	Element			
	Fe		Zn	
14	1.13	1.70	0.46	0.45
17	0.29	1.05	1.31	0.30
18	1.78	1.45	0.81	0.33
23	4.02	0.53	1.05	0.21
24	2.24	1.00	0.67	0.47
26	0.48	1.36	0.40	0.94
27	1.11	1.04	0.29	0.84
30	0.95	1.45	0.81	0.77
33	1.06	0.92	0.43	0.35
34	1.09	1.10	0.44	0.48
$\bar{x}$	1.42	1.16	0.67	0.51
$\lvert\Sigma d\rvert$	0.26		0.15	
$S_{\bar{d}}$	0.410		0.162	
$\lvert t\rvert$	0.622		0.944	

Notes:

1) Figures represent µg Element/ml sample

2) Left column for each element contains data from February 1976

3) Right column for each element contains data from January 1977

4) $t_{[10].95} = 1.83$

Table 24. Effect of storage on concentration of K in ashed *P. cuprodurans*.

	µg K/ml	
Sample	1975	1976
12	1060	3460
13	896	2370
16	825	2040
23	1320	3290
24	1020	2700
26	1450	3410
27	840	2050
$\bar{x}$	1050	2760
$\lvert\Sigma d\rvert$	1700	
$S_{\bar{d}}$	166	
$^1\lvert t\rvert$	10.2	

Notes:

1) $t_{[6].95} = 1.94$

To investigate this possibility, I prepared fresh KCl standards and compared them with the commercial reagent. I observed no difference, indicating that instrument alignment was involved.

A final consideration for interpreting the K data from Table 23 was that I had obtained the earlier values during the same analytical run as the 1976 values presented in Table 24. This information all indicated that the elemental composition of the samples did not change during storage. I concluded that samples from one analytical run should be used as standards for subsequent runs. As a second precaution, to ensure data comparability, I recommended using a single analytical run to assay all samples generated by an experiment although this may have meant storing some samples for several months.

Development of Wash Procedure

I have indicated the requirement for a washing step in the preceeding sections. The preliminary data suggested that Q2W provided as effective a wash as any of the buffered solutions. Furthermore, it was the only wash that did not contribute either organic or inorganic chemicals to the system. However, if Q2W had caused substantially more cell lysis or death than other wash solutions, the advantage gained by no contamination would have been off-set by loss of cell chemistry.

To determine the effect of washing and centrifugation on P. cuprodurans, I grew the culture in one-liter of modified

2216E in a two-liter Fernbach flask at 30 C and 200 rpm. At 18 h, I removed a 0.1 ml sample for spread-plating and centrifuged the culture at 1.6 x 10^4 x g for 10 min. I centrifuged the bacteria in six 300-ml polycarbonate centrifuge bottles and segregated the bottles into two treatment groups. After the initial centrifugation, I pooled pellets from each group forming two samples. I accomplished initial pooling by resuspending each pellet with the appropriate wash solution to effect transfer from one centrifuge bottle to another.

I washed one sample from each experiment with artificial seawater at a salinity of 26.5 ppt and washed the other sample with either Q2W of 0.5 N ammonium formate by resuspending manually the pellet in 100 ml wash reagent. After washing, I titered each sample by viable count and photographed a wet mount of each sample. I also obtained photomicrographs after centrifugation and before washing. I repeated this centrifugation-washing procedure two more times for each experiment and completed three experiments comparing Q2W and artificial seawater washes and one experiment comparing ammonium formate and artificial seawater washes.

Observing the change in A_{260} and A_{280} of the supernate during three successive washes, there was no reproducible UV absorbancy pattern among three replications of the experiments. One problem was that even after 15 min at 1.8 x 10^4 x g the second and third pellets had fluffy surfaces. This observation suggested that a fraction of the population after washing had a lower bouyant density than the

bulk of the cells. The viability of the UV absorbancy intensities might have been due to differential extrusion or diffusion of biomolecules from the bacteria during washing. Data from continuous centrifugation experiments (below) also suggested this possibility.

The photomicrographs (Fig. 7 and 8) provided no evidence that artificial seawater, Q2W or ammonium formate stressed P. cuprodurans sufficiently to cause more than 10 % of the cell population to become morphologically aberrant. I based this calculation on the assumption that in order to see one bacterium per field, there must be at least 4×10^6 cells/ml. Given a viable count of approximately 5×10^8 CFU/ml, it seems unlikely that stressed cells represented a significant fraction of the population unless several involuted bacteria appear in each field.

The viable counts of P. cuprodurans in these washes indicated more substantial cell lysis (Table 25) than I observed microscopically. CFU/ml decreased substantially after the initial wash. Additional viable titer decreases occurred for artificial seawater and ammonium formate washed cells after the second resuspension. Viable titer continued to decrease after a third wash in ammonium formate.

A weakness of viable bacterial counting was that small clusters of bacteria gave rise to single colonies leading to an underestimation of viable titer. Dark field observations (Fig. 9) of bacterial aggregates remaining after agitation indicated that a considerable fraction of the apparent loss in viability was due to incomplete disaggregation for discrete

Fig. 7. Effect of washing on P. cuprdurans morphology. a) P. cuprodurans prior to harvesting. b) after centrifugation and resuspension in Lyman and Fleming's filtered seawater at 25 ppt salinity, c) after centrifugation and resuspension in Q2W, d) after second resuspension in Q2W, e) after second resuspension in Q2W, f) after third resuspension in Lyman and Fleming's artifical seawater, g) after third resuspension in Q2W. Magnification 1,800 x.

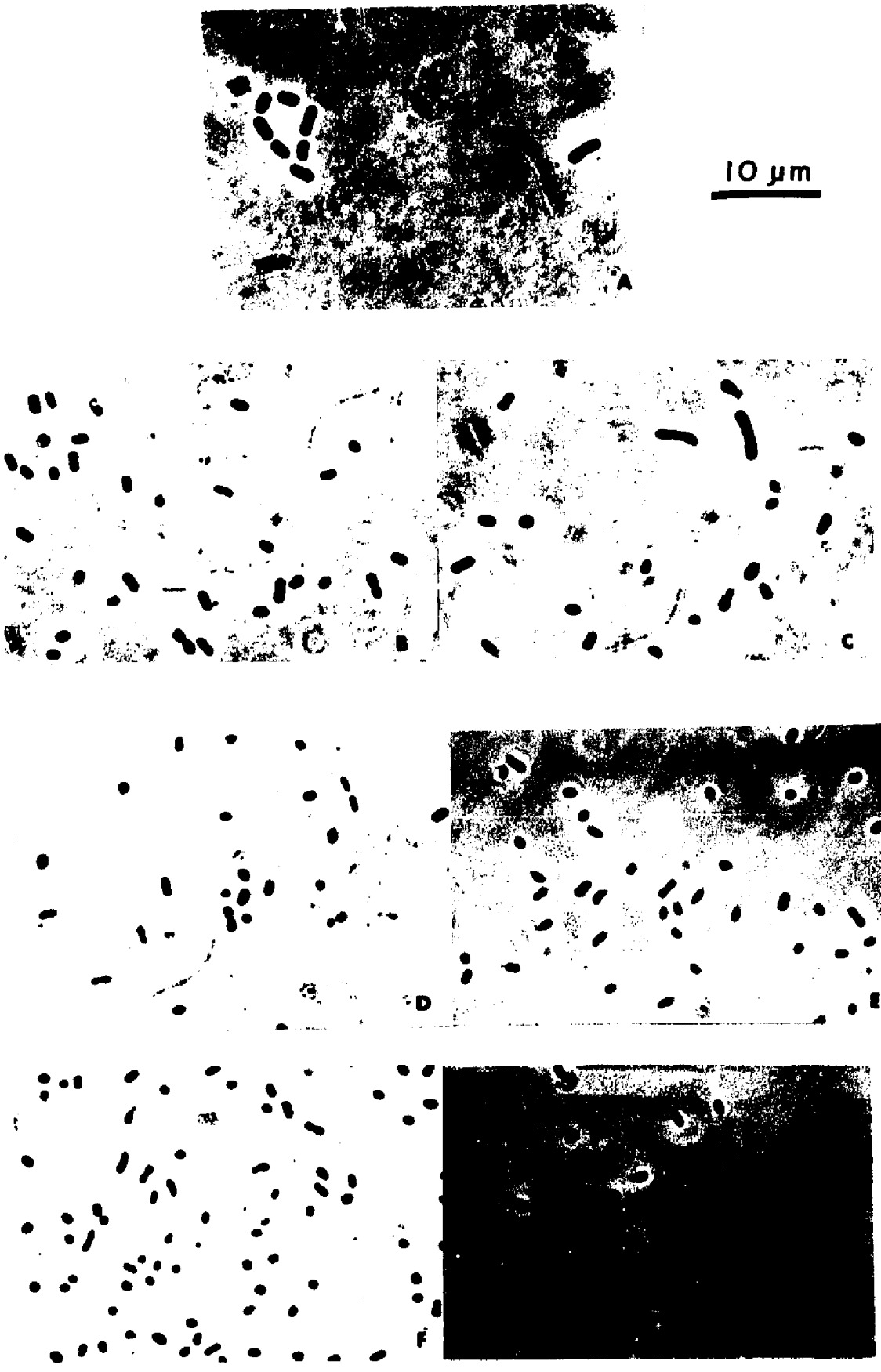
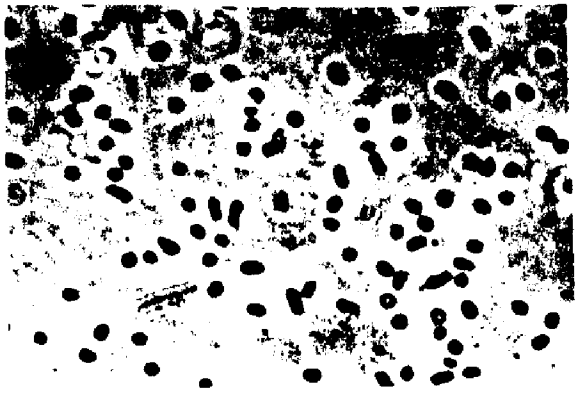

10 µm
A
B
C
D
E
F

Fig. 8. Effect of washing on P. cuprodurans morphology. a) after one resuspension in 0.5 N NH_4COOH, b) after two resuspensions in 0.5 N NH_4COOH, c) after three resuspensions in 0.5 N NH_4COOH. Magnification 1,800 x.



A

B

C

10 µm

Table 25. *P. cuprodurans* survival following successive washes during harvest.

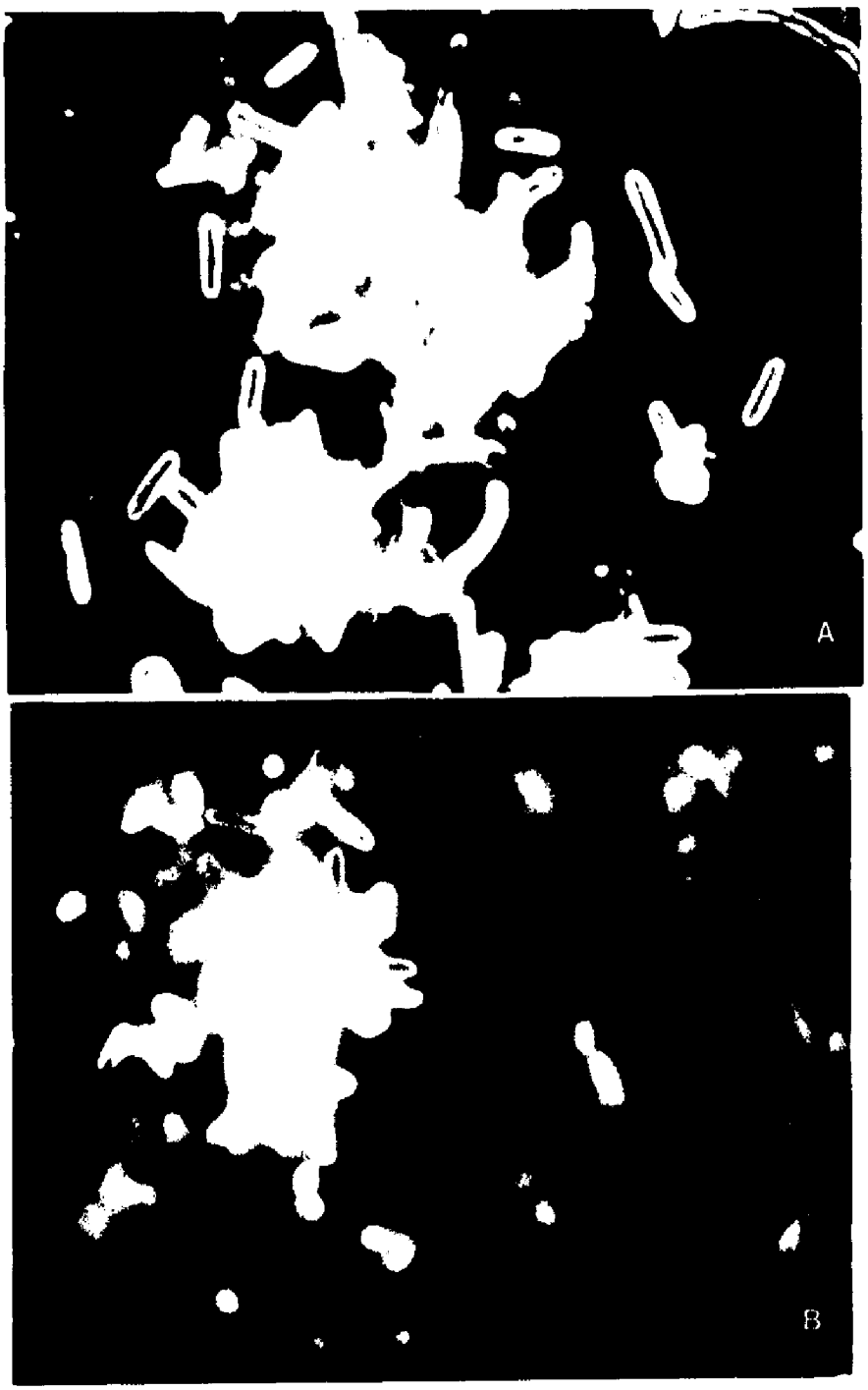
[1]Resuspension	Wash		
	Survival as Percent Pre-Harvest Viable Count Titer		
	[2]Artifical Seawater	Deionized Water	0.5N Ammonium Formate
1	63.9	10.7	6.25
2	10.1	12.0	3.99
3	8.62	8.85	0.76

Notes:

1) Samples for viable counts were taken after pellet was resuspended in wash.

2) Salinity was adjusted to 25.0 $\pm$ 0.5 ppt.

Fig. 9. P. cuprodurans after a) one resuspension in Q2W and b) after two resuspensions in Q2W. Dark field illumination. Magnification 5,700 X.



1 µm

CFU's. The clumps were small enough to provide access to wash solutions and yet large enough so each CFU on spread plates may represent more than one cell. Thus, viability loss may be somewhat misrepresented in Table 25.

Resuspending the bacteria was another potential source of experimental variation. During preliminary experiments, I resuspended pellets by manual agitation until the pellet was no longer visible, a period of about 10 min. To eliminate this inconsistent source of variation, I incorporated a timed, mechanical agitator.

Another factor which may have contributed to cellular disappearance during washing was sheer-stress due to agitation with the Vibromixer. To investigate this possibility, I grew P. cuprodurans in 100 ml of modified 2216E broth in a 250 ml Erlenmeyer flask at 30 C and 200 rpm. At 13 h, I diluted the broth 1:10 by transferring the Erlenmeyer flask's contents to a two-liter Fernbach flask, containing 900 ml sterile medium at 4 C. After thoroughly mixing the diluent and original broths, I drew a 0.1 ml sample for spread plating and removed two 100 ml aliquants from the diluted culture, transferred them to 250 ml polycarbonate centrifuge bottles and set them in ice, designating the aliquants as test and control. I agitated the test aliquant with a Vibromixer for 10 min and left the control undisturbed, drawing a sample from each aliquant for spread plating at the end of the agitation period. I removed four more pairs of samples in succession from the two-liter Fernbach flask, subjecting each pair to the treatment described above,

removing a sample for spread plating from the Fernbach flask before removing each pair of aliquants. The viable titer decreased from log CFU/ml 8.19 to 7.76 during the 1.5 h that lapsed between initiation of the first removal and the final removal (Table 26). I observed this decrease in the bulk sample and control series but not in the test series.

I defined S_c and S_t as:

$$\frac{\text{(CFU/ml) control after}}{\text{CFU/ml bulk prior}} \quad \text{and} \quad \frac{\text{(CFU/ml) test after}}{\text{(CFU/ml) bulk prior}}$$

respectively. Δ, which was S_t/S_c was approximately unity for all five replicates.

Agitation did not have an affect on P. cuprodurans viability. Photomicrographic data presented above provided no indication that the bacteria were subjected to sufficient stress to cause apparent morphological changes. I had observed incomplete disaggregation which supported the conclusion that the viable titer decrease was a clumping phenomenon. The washing technique was sufficiently gentle not to cause major losses of cell associated elements. I based the ashing procedure in Materials and Methods on these observations.

Development of Ash Technique

The two most widely employed ash techniques were wet digestion in redistilled HNO_3 and dry oxidation in a muffle furnace. To determine what effect these techniques have on subsequent analyses, I designed a 2 x 2 factorial experiment. Factors considered were ashing

Table 26. Survival of Pseudomonas cuprodurans after 10 min agitation with a Vibromixer.

Experimental Run	Log CFU/ml					
	Prior to Agitation	Control	After Agitation	^1S_c	^2S_t	$^3\Delta$
1	8.19	8.41	7.96	1.03	0.97	0.94
2	8.06	7.91	7.96	0.98	0.99	1.01
3	8.01	7.97	8.06	1.00	1.01	1.01
4	7.93	7.85	8.20	0.99	1.03	1.04
5	7.76	7.78	8.00	1.00	1.03	1.03
			$\bar{Y}$	1.00	1.01	1.00
			x	0.00	0.00	0.00

Notes:

1) S_c = (CFU/ml) control after/(CFU/ml) bulk prior

2) S_t = (CFU/ml) test after/ (CFU/ml) bulk prior

3) $\Delta = S_t/S_c$

technique and experimental replication.

I grew E. coli on filter-sterilized Nutrient Broth in a 7-liter Microferm fermentor at 37 C aerated with paddles rotating at 200 rpm which dispered air pumped into the vessel at 1.5 liters/min, and harvested cells by centrifuging at 1.8×10^4 x g in 250 ml polycarbonate bottles. I combined the six pellets in a tared teflon beaker, dried them at 110 C without washing, and used an agate mortar and pestle to grind the dry material to a fine powder. I distributed the powder (approximately 100 mg) among six tared silica or porcelain crucibles and six tared teflon beakers and repeated the experiment three times.

I employed two ash techniques. Wet-ashing samples in teflon beakers in redistilled nitric acid until the residue was pale yellow and crystalline, I allowed the acid to evaporate to dryness then cooled the samples in a desiccator over silica gel and determined ash yields by computing

$$\left(\frac{\text{mg Ash}}{\text{mg Dry Weight}}\right) \times 100.$$

I dissolved samples in 2.0 ml 6 N redistilled HCl, transferred them to 25 ml volumetric flasks, brought them to volume with Q2W rinsings from the digestion beaker, and transferred them to polypropylene vials for storage at -4 C.

I dry-ashed samples in the crucibles by heating at 450-500 C in a muffle furnace (Jelrus Technical Products Corp., New Hyde Park, N.Y.) until only a light-colored residue remained. I cooled samples in desiccators over silica gel desiccant and weighed them to determine ash yields. To

ensure that ashing had been complete, I redissolved ash residues in redistilled HNO_3 to evaporate to dryness and replaced it with 2.0 ml redistilled HCl. I transferred the samples to 25 ml volumetric flasks, and brought them to volume with Q2W rinsings from the crucibles before transferring them to polypropylene vials for storage at -4 C.

Before performing atomic absorption spectroscopy on the samples, I compared wet and dry ash yields among the three experiments (Table 27). Dry-ash yields within an experiment were consistently more precise than wet-ash yields from the same experiment. This was due to the contribution of HNO_3 to the ash weight of wet digested samples. However, dry-ash yield variation among experiments was more than four times the wet-ashed yield variation. This suggested that the more precise dry-ashing technique permitted finer resolution of differences among experiments.

This hypothesis was substantiated by the ANOVA summarized in Table 28. As anticipated, the ash technique significantly affected the ash yield. Variations due to experiment replications were also significant at the 95 % confidence level. The effect of each ash technique was consistent among replicates.

Persuing the comparison one more step, I analyzed the same samples from two E. coli experiments for P, K, Na, Ca, Mg, Fe, and Zn (Table 29); and computed analysis of variance for the data (Table 30). P, Na, and Mg concentrations varied significantly between experiments. Ash treatments significantly affected Na, K, and Zn concentrations. An interaction

Table 27. Precision of wet and dry ash techniques for E. coli grown on Nutrient Broth at 37 C.

	[1]Dry Ash			Wet Ash		
Experiment	1	2	3	1	2	3
Subsample						
1	4.04	5.29	10.34	11.01	21.70	22.28
2	3.98	6.47	10.00	12.54	10.57	19.10
3	4.01	6.65	9.48	17.16	17.83	14.91
4	4.06	4.73	9.52	16.54	21.16	19.80
5	3.98	6.61	9.08	21.66	16.64	17.30
6	3.91	5.52	9.02	14.65	19.42	22.02
$\bar{x}_i$	4.00	5.88	9.57	15.59	19.39	19.24
CV %	1.33	13.78	5.51	24.25	9.90	14.66
Grand Mean	6.48			18.07		
Grand CV %	35.7			9.72		

Notes:

1) Ash Yield = $\frac{\text{mg Ash}}{\text{mg Dry Wt}}$ x 100

Table 28. Summary of analysis of variance for effects of experimental replication and ash treatment on ash as a fraction of dry weight.

Source of Variation	SS	df	MS	[1]F
Replication	32.2	1	32.2	8.4
Ash Technique	1504.6	1	1504.6	329.8
Experiment x Technique	1.6	1	1.6	0.4
Error	153.2	40	3.8	---
Total	1750.2	43	---	---

Notes:

1) $F_{.95\ (1,40)} = 1.69$

Table 29. Factorial analysis of effects of ash technique and experiment on concentrations (ppm dry weight) of P, K, Na, Ca, Mg, Fe and Zn in E. coli grown on Nutrient Broth at 37 C.

Element	Experiment	Ash Technique: Dry $\overline{ppm}$ [1]	Dry Range [2]	Wet $\overline{ppm}$	Wet Range
P	1	2.54×10^4	$2.47 - 2.67 \times 10^4$	2.55×10^4	$2.42 - 2.63 \times 10^4$
	2	1.22×10^4	$0.94 - 1.44 \times 10^4$	1.62×10^4	$0.99 - 1.96 \times 10^4$
K	1	2.02×10^4	$1.13 - 3.05 \times 10^4$	4.06×10^4	$3.66 - 4.40 \times 10^4$
	2	1.24×10^4	$0.90 - 1.47 \times 10^4$	4.19×10^4	$2.59 - 4.92 \times 10^4$
Na	1	3.28×10^3	$3.11 - 3.40 \times 10^3$	3.43×10^3	$3.22 - 3.56 \times 10^3$
	2	5.93×10^3	$5.28 - 6.69 \times 10^3$	3.52×10^3	$3.35 - 3.59 \times 10^3$
Ca	1	1.51×10^3	$1.36 - 1.70 \times 10^3$	1.42×10^3	$1.31 - 1.60 \times 10^3$
	2	1.78×10^3	$1.04 - 2.23 \times 10^3$	1.35×10^3	$0.67 - 2.18 \times 10^3$
Mg	1	2.77×10^3	$2.61 - 2.88 \times 10^3$	2.92×10^3	$2.83 - 3.02 \times 10^3$
	2	1.64×10^3	$1.24 - 1.80 \times 10^3$	1.64×10^3	$1.05 - 1.96 \times 10^3$
Fe	1	210	115 - 292	192	82.9 - 267
	2	249	83.8 - 496	207	112 - 263
Zn	1	93.8	65.8 - 118	189	69.7 - 434
	2	113	64.8 - 161	140	94.7 - 229

Notes:

1) $\overline{ppm}$ was mean for 6 subsamples

2) Range was min and max ppm among the 6 subsamples

Table 30. Summary of ANOVA for effects of ash technique and experiment replication on P, K, Na, Ca, Mg, Fe and Zn concentrations in E. coli grown on Nutrient Broth at 37 C.

Source of Variation	Element													
	P		Na		Mg		Zn		K		Ca		Fe	
	f_{obs}[1,2]	sig[3]	f_{obs}	sig	f_{obs}	sig	f_{obs}	sig	f_{obs}	sig	f_{obs}	sig	f_{obs}	sig
Experiment	246	yes	8.73	yes	240	yes	0.37	no	0.00	no	1.00	no	1.00	no
Ash Technique	0.00	no	5.50	yes	0.00	no	4.48	yes	121	yes	1.00	no	0.00	no
Experiment x Ash Technique	1.60	no	7.34	yes	0.00	no	1.17	no	6.57	yes	2.00	no	1.00	no

Notes:

1) $f_{obs} = \frac{MS_{source\ of\ error}}{MS_{error}}$

2) $f_{.95\ (1,23)} = 4.30$; if $f < 4.30$, effect of factor on variation was not significant at 95 %.

3) sig = significant

effect between experiment replication and ash technique caused variation between Na and K concentrations in samples.

The anomalously high Na concentrations in dry-ashed subsamples from experiment two accounted for 74 % of the overall variation among Na samples and were responsible for the high f values in the ANOVA for Na. K in dry-ashed samples varied by approximately 50 % between experiments. Moreover, K concentrations in dry-ashed samples were lower than in wet-ashed samples. From these two observations, I concluded that K was volatilized during dry ashing.

The essential point made by Tables 28 through 30 was that within an experiment, data from subsamples was sufficiently precise to permit resolution of procedural variations. I could differentiate replicate fermentor experiments on the basis of the elemental composition of the dried bacterial samples. Caution in interpretation of the results was indicated since I was only able to ash the six subsamples from a particular fermentor run and two controls in a single muffle furnace cycle. Temperature control on the furnace was only accurate to $\pm$ 50 C, and as demonstrated in Table 29, ash yield variation among runs was considerable. I overcame this disadvantage by replacing the small muffle furnace with a Thermolyne Model 1700 furnace (Thermolyne Sybron Corp., Dubuque, Iowa) with a 40 crucible capacity. In order to obtain ash yield data, I adopted dry ashing as the primary ash treatment, concluding that secondary treatment with redistilled HNO_3 was necessary for solubilizing the ash residue once ash yields were determined.

As dry ashing had several advantages over wet ashing, I selected it as the standard technique for preparing bacteria for atomic absorption spectrophotometry. I still had to minimize variation among ash yields from different muffle furnace batches. The previous experiment indicated the need for tighter control of combustion times and temperature. I conducted an experiment to determine the effect of more rigorous control of the intensity and duration of the oxidation period.

I cultivated E. coli in the Microferm fermentor and harvested cells by the procedure outlined for the preceeding experiment. I ashed samples for 43 h at 500 C. As in the previous experiment, I ashed six equivalent subsamples during each combustion cycle. I repeated the experiment, then compared ash yields from the first and second experiments (Table 31). The yield data demonstrated that I could obtain ash reproducibly by the optimized technique as described in the Materials and Methods section. To determine whether elemental analyses for these samples were also reproducible, I also compared data for Na, K, Ca, Mg, Fe, Zn, and Cu using the paired t-test (Table 32). At the 95 % confidence level, the critical value for t was 2.02, given five degrees of freedom (df). On this basis, between the two ashing runs, there were no significant concentration differences for any of the elements analyzed. Variation among subsamples ranged from 8.06 % for K to 63.6 % for Mg.

One potential source of variation among subsamples

Table 31. Summary statistics for ash yields of E. coli samples wrapped in Whatman #42 filter paper.

(mg Ash / mg Dry) x 100

	Run 1	Run 2
	5.16	5.64
	5.18	5.56
	5.37	5.47
	5.22	5.51
	5.63	4.86
	5.66	4.91
Total	32.22	31.95
Mean	5.37	5.32
s	0.226	0.337
C.V (%)	4.20	6.28

Grand Total	64.17
Mean	5.35
s	0.279
C.V. (%)	5.22

$\Sigma d_i = -0.80$

$s_{d_i} = 0.977$

$|t| = 0.824$; $t_{[5].95} = 2.02$

Table 32. Elemental composition of *E. coli*; comparison of analyses from two experiments.

	Element						
Experiment	Na	K	Ca	Mg	Fe	Zn	Cu
1	[1]2150	4420	1890	1400	145	50.3	41.1
2	2180	4570	2790	1100	135	42.3	58.0
Σd	-26.0	-155	-906	301	9.57	8.13	-16.9
S_{xy}	354.7	147.2	637.4	558.8	34.17	5.35	11.18
[2]$\lvert t \rvert_{obs}$	0.07	1.05	1.42	0.53	0.28	1.51	1.51
[3]$\bar{G}$	2160	4490	2340	1250	140	46.3	49.6
CV_G%	27.3	8.06	48.7	63.6	38.8	21.3	39.7

Notes:

1) Figures represent means of 6 subsamples (ppm)

2) $t_{[.95]}$ for 5 degrees of freedom = 2.02

3) $\bar{G}$ = mean for 12 subsamples of element X; S_x and CV_x were computed with $\bar{G}$ (s_x not presented).

was sequestering of ions in the insoluble fraction. Even after the combined dry-wet ash procedure, there remained a small, insoluble fraction. To determine the composition of this residue, I filtered samples prepared for atomic absorption spectroscopy through a 0.2 μm pore size membrane and analyzed them by EDAX. The amorphous residue from a 140 mg (dry weight) sample coated the membrane (Fig. 10a). Energy dispersive x-ray analysis revealed four prominent peaks (Fig. 10b). In order of increasing energy, the peaks were Na, 1.041 KeV; Mg, 1.253 KeV; Si, 1.739 KeV; and P, 2.013 KeV. (O, Fe and traces of K were also detected). Mg was localized in some of the particles and Si in others. P was distributed evenly throughout the residue.

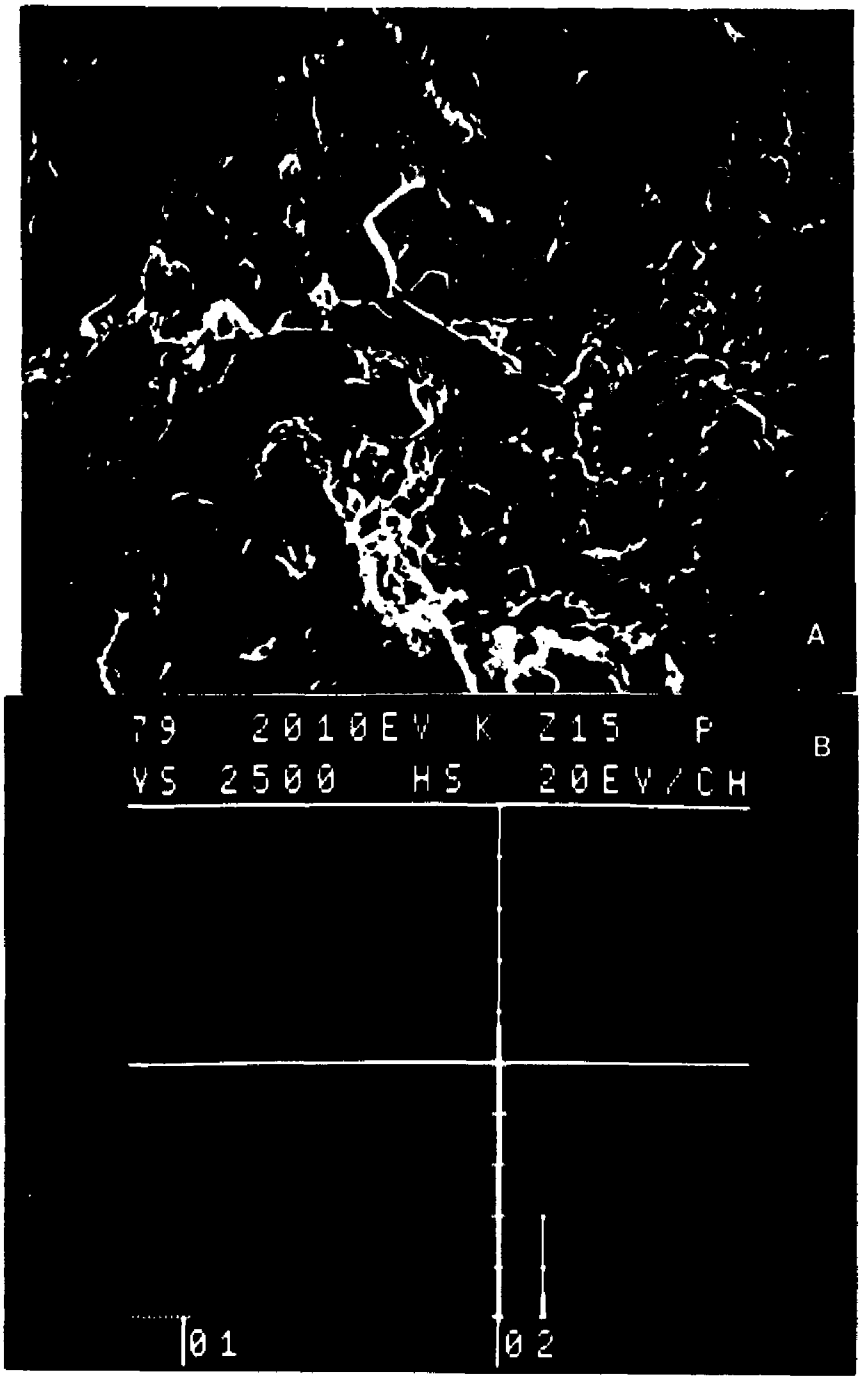
Although I was never able to solubilize a fraction of the ash, I considered the ash and sample preparation techniques to be standardized. I shall discuss my rationalization for this in perspective with the methodologies employed by other investigators. To minimize variability due to nebulization and atomization of undissolved particles, I filtered samples through Whatman #42 paper as I dispensed them from crucibles to 25 ml volumetric flasks, assuming that any loss of elements would be consistent and that precision was more important than absolute accuracy. Again, I will elaborate upon the rational for this decision in the discussion.

Growth Measurements

Having established a standard procedure for harvesting, washing, ashing, storing, and analyzing samples, I was able

Fig. 10. a) Scanning electron micrograph of acid insoluble residue of ashed P. cuprodurans. Magnification 2,300 x. b) Energy dispersive x-ray spectrum of insoluble residue from 0.75 - 2.75 KeV analyzed at 10 KV.

5 µm



to return to the cultivation problem. I theorized that the most suitable means of obtaining three to five g dry weight quantities of homogeneous bacterial sample was to grow and harvest the bacteria in a continuous cultivation system. In order to monitor P. cuprodurans population density in the continuous cultivation apparatus, I had to select a suitable parameter.

By comparing four parameters against a nine step two-fold dilution series, I obtained data regarding the accuracy and precision of each parameter. I transformed the data to $\log_2$ to facilitate determination of a correlation between each parameter and the concentration of bacteria in solution. I expected parameters which accurately measured population densities to yield regression lines with slopes and regression coefficients equalling unity. I anticipated that precise parameters would have low S_{xy} values (minimal variation about the best fit line defined by the regression equation).

The data for $\log_2$ X as a function of $\log_2$ dilution, where X was the assayed parameter, was summarized in Table 33. Viability was correlated to dilution through the greatest number of steps, followed successively by protein concentration, A_{420}, and dry weight. Although regression coefficients reflected close correlation between individual data and regression equations for all four parameters, only viable titers and A_{420} had slopes approximating unity. Fig. 11 demonstrated that the turbidimetric data was much more precise than the viable count data.

Table 33. Comparison of parameters for monitoring P. cuprodurans population densities.

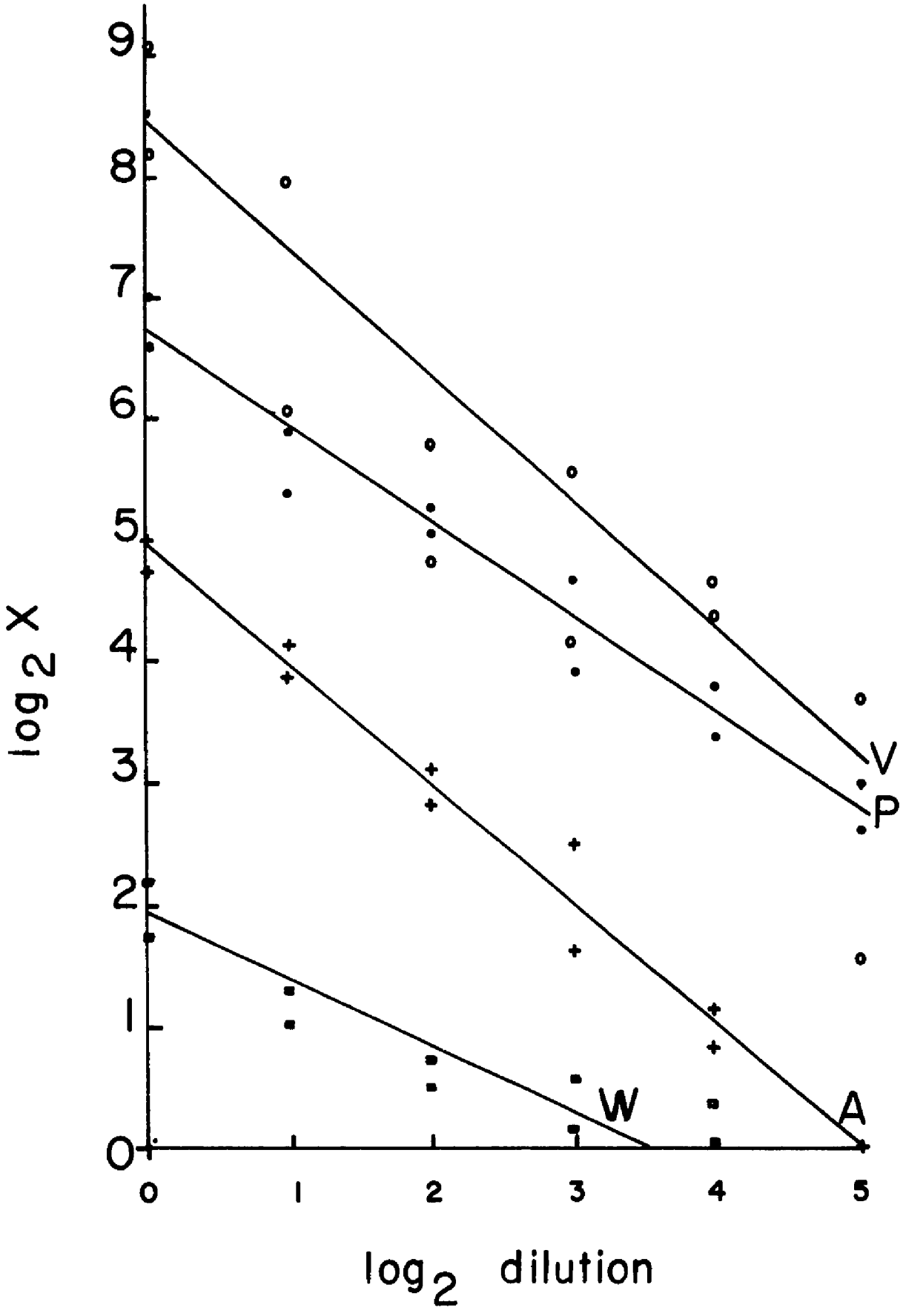
Dilution	$\log_2$ Parameter Viability CFU/ml	Protein µg/ml	A_{420} (logA)+10	Dry Wt mg/ml
9	[1]--	0.11	3.33	0.60
8	[2]20.2	1.43	5.80	0.76
7	21.0	1.31	4.71	0.86
6	22.1	1.97	6.37	0.56
5	22.9	3.05	5.76	0.74
4	24.9	3.64	6.66	1.00
3	25.2	4.47	8.04	1.09
2	25.7	5.27	8.85	1.35
1	27.1	5.93	9.95	1.94
0	28.1	7.06	10.8	2.69
[3]slope	-1.07	-0.79	-1.03	-0.54
regression coefficient	0.97	0.99	1.00	0.98
S_{xy}	0.66	0.22	0.17	0.14

Notes: 1) Values above line did not vary with dilution, and were not included in calculation of regression.

2) Values represent mean of three samples.

3) Slope, regression coefficient and S_{xy} are means for three regression equations.

Fig. 11. Comparison of best fit curves for $\Delta \log_2 X$ vs. $\log_2$ dilution, where X is population density parameter: open circles, viability; closed circles, protein concentration; crosses, A_{420}; open squares, dry weight. Vertical distance between markers is range of measurements at that dilution.


9
8
7
6
5
4
3
2
1
0
log2 X
0
1
2
3
4
5
log2 dilution
V
P
W
A

I grew P. cuprodurans in modified 2216E of 12.8 ppt salinity at 15 C and 245 rpm and compared A_{420} with viable titer during growth. The data (Table 34; Fig. 12) demonstrated that the correlation between the two parameters was only valid during logrithmic growth.

Of the four parameters compared, I considered A_{420} best-suited for use in monitoring population densities during the continuous cultivation of P. cuprodurans. For absorbance, time lag between sampling and data acquisition was minimal. Furthermore, A_{420} was closely correlated to both viable titer and dilution. Although viable titer was more sensitive than A_{420}, I considered the delay between sampling and data acquisition unacceptable for continuous monitoring of population densities.

Taxonomy of P. cuprodurans

P. cuprodurans had been stored on a modified 2216E agar slant at 4 C for three years before I began my investigations. Although the bacterium had the same morphology as described by McCarthy (1971), it was possible that other phenotypic changes had occurred. Also, McCarthy's taxonomic report did not contain antibiotic sensitivity, mole % (G + C), or nutrient requirement data. As I intended to grow P. cuprodurans in a continuous cultivation apparatus, I considered it necessary to duplicate and expand McCarthy's taxonomic investigation.

Table 34. P. cuprodurans growth curve at 15 C, 275 rpm agitation, in autoclaved modified 2216E (S = 12.81 ppt) broth medium.

Time (hr)	Replicas	$\bar{A}_{420}$	C.V.	[1] $\overline{CFU}$	C.V.	log $\overline{CFU}$/ml	[2] $\overline{pH}$	C.V.
0	6	0.020	2.098	9.87×10^5	0.527	5.99	7.54	0.001
12	3	0.143	0.145	7.27×10^6	0.596	6.86		
13	3	0.06	0.712				7.53	0.000
14	6	0.19	0.539	1.43×10^7	0.524	7.16	7.44	0.004
16	6	0.41	0.632	1.01×10^7	1.710	7.00	7.35	0.007
18	6	0.68	0.527	8.33×10^7	0.139	7.92	7.22	0.010
20	6	0.88	0.369	6.33×10^7	0.379	7.80	7.17	0.008
22	6	1.17	0.277	2.00×10^8	0.477	8.30	7.04	0.012
24	6	1.46	0.243	3.90×10^8	0.462	8.59	7.01	0.002
26	4	1.70	0.057				[3] 7.74	0.006
85	3	1.64	0.009					
118	3	1.57	0.013				8.17	0.002

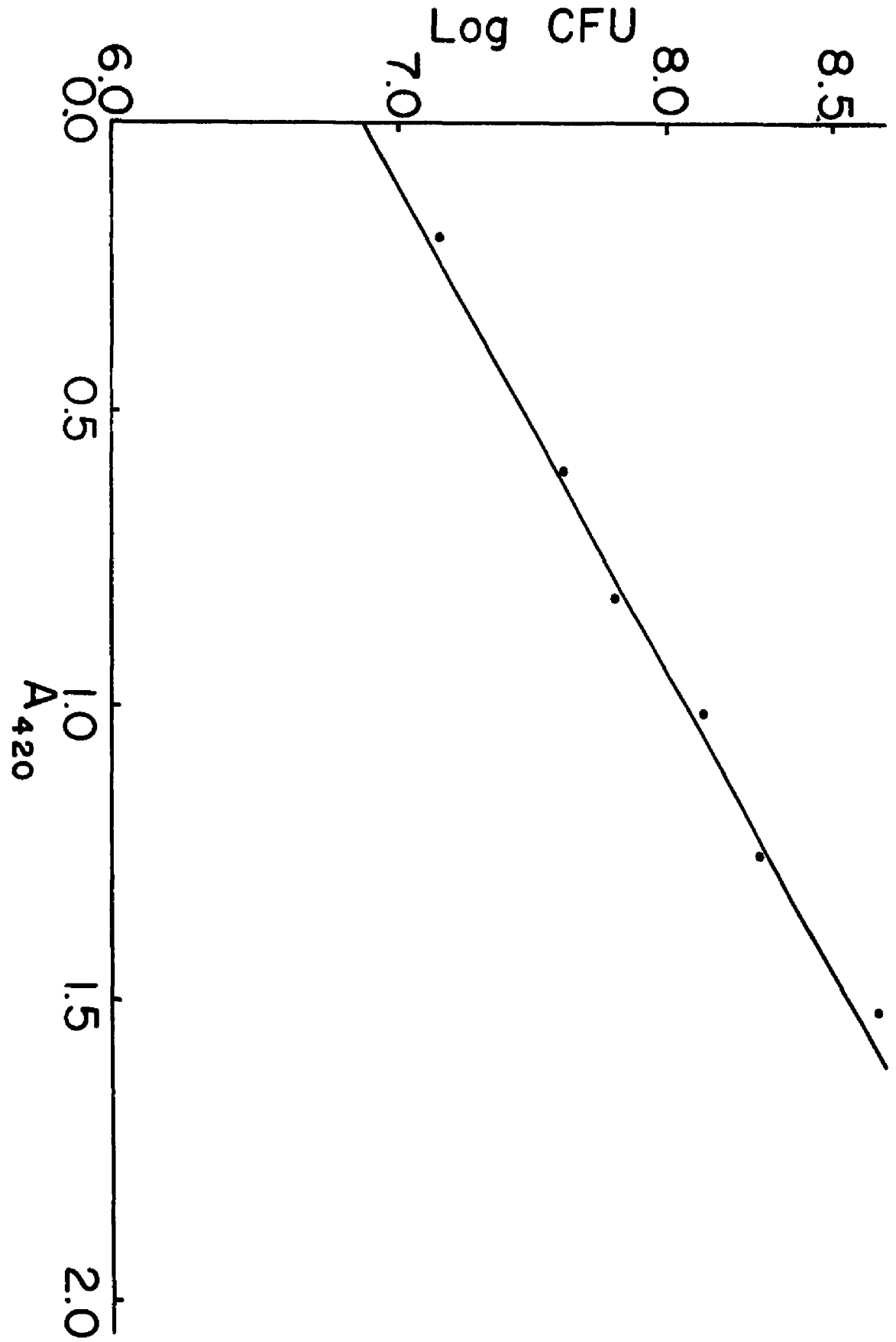
Notes:

1) CFU data is based on three replications.

2) pH data is based on three replications.

3) This data point was taken at 39 h.

Fig. 12. Relationship between viable counts and A_{420} for P. cuprodurans grown on autoclaved modified 2216E; 12.8 ppt salinity. The comparison is only meaningful during logrithmic growth.


Log CFU
6.0
7.0
8.0
8.5
0.0
0.5
1.0
1.5
2.0
A_{420}

Morphology

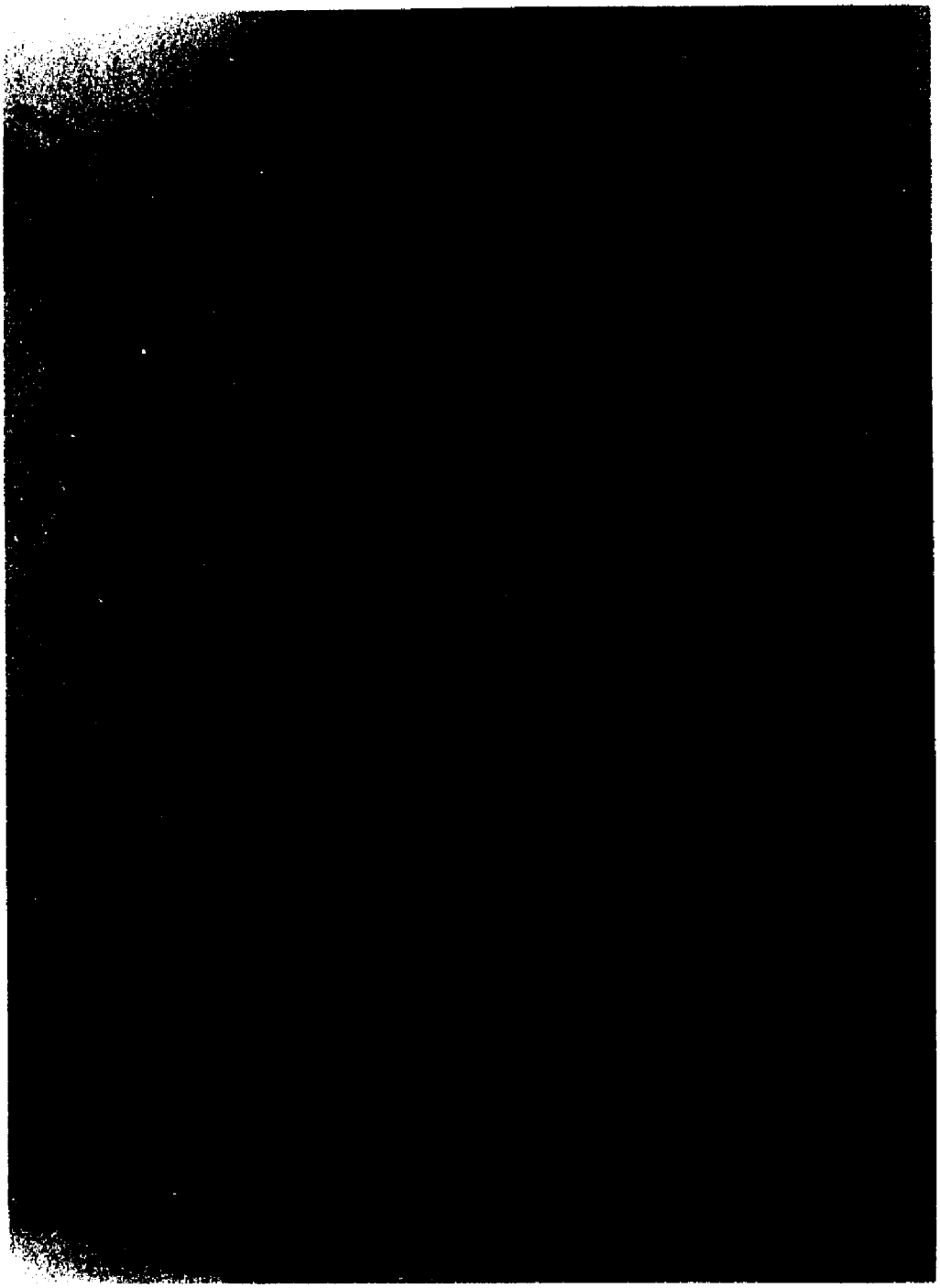
I observed P. cuprodurans was a 0.5 - 0.75 x 1.0 - 1.2 μm polarly flagellated rod (Fig. 13) that appeared as individuals, pairs or chains of four to eight cells (Fig. 14). Wet mount observations by phase contrast microscopy indicated that the bacterium was motile and uniformly phase dense. P. cuprodurans demonstrated motility on soft agar streak plates. The bacterium was gram-negative, non-acid fast, non-sporulating, and did not contain fat droplets.

Colony morphology on extract, trypticase-glucose extract and potato-dextrose agars was summarized in Table 35. I did not observe chromogenesis on potato-dextrose agar. After three days, I observed profuse growth in extract and modified 2216E broths. For both broths, turbidity was uniform, the deposit was moderate, flocculant and easily disintegrated. There was no apparent surface growth. P. cuprodurans produced a yellow-green, fluorescent pigment in pseudomonas agar F, but not in agar P, indicating fluorescein but no pyocyanin production (King, Ward, and Raney, 1954).

Nutrition and Physiology

I listed the results of physiological tests in Table 36. P. cuprodurans did not grow on nicotine agar. Data for carbohydrate utilization depended upon the base medium used. In phenol red broth, P. cuprodurans grew in arabinose, dextrin, fructose, galactose, glucose, inosotol, lactose, maltose, mannitol, mannose, rhamnose, ribose, salicin, sucrose, and xylose. The culture produced acid without gas in each of the

Fig. 13. P. cuprodurans. Electron micrograph of negatively stained cell, demonstrating single polar flagellum 38,000x.



0.5 μm

Fig. 14. P. cuprodurans. Electron micrograph of negatively stained preparation. Cells appear as individuals, pairs and chains. 18,000x.

1.0μm

Table 35. P. cuprodurans colony morphology.

[1]TRAIT	MEDIUM		
	Extract Agar	Trypticase - Glucose	Potato Dextrose Agar
Shape	circular	circular	circular
Size	1.2 mm	2-5 mm	1 mm
Chromogenesis	diffusive, fluorescent yellow pigment	diffusive, fluorescent yellow pigment	none
Opacity	opaque	opaque	opaque
Elevation	raised	raised	raised
Surface	glistening	glistening	glistening
Margin	entire	entire	entire
Consistancy	butyrous	butyrous	butyrous
Color	cream	cream	cream

Note:

1) Observed at 36 h

Table 36. Physiological characteristics of P. cuprodurans.

TEST	REACTION
Gelatin liquefication	[1]N
Starch hydrolysis	N
Indole production	N
H_2S production	N
Citrate utilization	[2]P
Ammonium-glucose utilization	P
Nitrate-glucose utilization	P
Creatine as nitrogen and carbon source	N
Creatine as carbon source	N
Cellulose utilization	N
Thiotone utilization in LFSW (25 ppt S) in distilled water	P N
Acetylmethylcarbinol production	N
Catalase	P
Oxidase	P

Notes:

1) Negative reaction

2) Positive reaction

other carbohydrates. In synthetic base broth, P. cuprodurans produced acid without gas in arabinose, dextrin, fructose, galactose, glucose, maltose, mannose, and xylose.

The auxanographic study demonstrated that P. cuprodurans was prototrophic, growing on ammonium, glucose and salts medium.

Antibiotic Sensitivity

Table 37 summarizes data on the response of P. cuprodurans to 15 antibiotics. The organism was not sensitive to carbenicillin, gantrisin, Na-cephalothin, penicillin G, or oxytetracycline. The inhibition zone around streptomycin was hazy, suggesting that P. cuprodurans was somewhat resistant to this antibiotic.

Mole % (G + C)

Yields of purified DNA from 12 h cultures, grown in 500 ml modified 2216E broths at 20 C, averaged about 200 µg. Difficulties in disrupting and removing cell walls contributed to relatively low DNA yields. I had to use concentrations of lysozyme and sodium lauryl sulfate two to five times that called for in Marmur's (1961) procedure to lyse the cells completely. Marmur acknowledged this type of problem in his paper.

I determined the mole % of P. cuprodurans to be 63.2 $\pm$ ' ' at the 95 % confidence level. I converted melting temperatures to % (G + C) by Schildkraut and Liefson's (1965) formula:

Table 37. *P. cuprodurans* antibiotic sensitivity on marine agar.

ANTIBIOTIC	CONCENTRATION	DIAMETER OF INHIBITION ZONE (mm)
Ampicillin (Difco	10 mgc	0
Aureomycin (BBL, chlortetracycline)	50 mgc	12
Carbenicillin (Difco)	50 mgc	0
Demethylchlortetracycline (BBL)	30 mgc	12
Cephalothin (Difco)	30 mgc	32
Erythromycin (Difco)	15 mgc	14
Gantrisin (BBL)	1 mgc	0
Gentamycin (Schering)	5 mgc	11
Kanamycin (Difco)	30 mgc	13
Penicillin G (Difco)	10 u	0
Polymyxin B (Difco)	300 u	18
Streptomycin (Difco)	10 mgc	11
Terramycin (BBL, oxytetracycline)	5 mgc	0
Tetracycline (Difco)	30 mgc	10

$\Theta_{GC} = [\tan (70.077 + 3.22\ M_{Na})]\ (T_m - 175.95) + 260.34.$ Θ_{GC} was the mole % (G + C), T_m was the midpoint of the corrected hyperchromatic shift curve, and M_{Na} was the molarity of Na^+ in the solvent.

Ionic Requirements

P. cuprodurans required seawater medium for growth; the specific ionic requirements being summarized in Table 38. Na^+, Mg^{2+}, Ca^{2+}, K^+, and $SO_4 2-$ were required, while Br^-, F^-, BO_3^{3-}, Sr^{2+}, and $CO_3 2-$ were not. Absence of two or more of the latter five ions did not restrict growth.

Growth Rate as Function of Temperature

I grew P. cuprodurans at 7, 15, 20, 25, 30, 35 and 40 C in modified 2216E at 245 rpm in a waterbath shaker. The bacterium grew at all temperatures except 40 C. To facilitate graphic representation and specific growth rate computation, I transformed duplicate A_{420} values to duplicate $10 + \ln A_{420}$. The transformed values were all positive and greater than 1.0 (Tables 39 and 40).

I defined specific growth rate, μ', as:

$$\mu' = \frac{\Delta(\ln A + 10)}{dt}$$

where t was time in h. Since μ' was linear during exponential growth, I computed it as the slope of the regression equation describing the best fit line through the time interval during which P. cuprodurans grew exponentially. In Tables 39 and 40, I bounded the time interval containing the data I used to compute the regression equation with horizontal lines.

Table 38. Pseudomonas cuprodurans ionic requirements.

Component of LFSW Deleted	Growth After 24 h
NaCl	N[1]
$MgCl_2$	N
Na_2SO_4	N
$CaCl_2$	N
KCl	N
$NaHCO_3$	P[2]
KBr	P
NaF	P
KBr, H_3BO_3	P
KBR, $SrCl_2$	P
KBr, NaF	P
H_3BO_3, $SrCl_2$	P
H_3BO_3, NaF	P
$SrCl_2$, NaF	P
KBr, H_3BO_3, $SrCl_2$	P
KBr, H_3BO_3, NaF	P
KBr, H_3BO_3, $SrCl_2$, NaF	P
Glucose (Control)	N
All Salts (Control)	N

1) Negative - no turbidity

2) Positive - turbidity

Table 39. *P. cuprodurans* growth curve data for 7, 15 and 20 C.

	7 C				15 C				20 C			
Time (h)	A_{420}	ln A+10	A_{420}	ln A+10,	A_{420}	ln A+10	A_{420}	ln A+10,	A_{420}	ln A+10	A_{420}	ln A+10
0	0.00	--	0.00	--	0.00	--	0.00	--	0.04	6.78	0.00	6.09
3	--	--	--	--	0.02	6.09	0.02	6.09	0.07	7.34	0.09	7.59
4	--	--	--	--	--	--	--	--	0.09	7.59	0.10	7.70
5	--	--	--	--	0.12	7.91	0.13	7.94	0.12	7.88	0.13	7.96
5.5	--	--	--	--	--	--	--	--	0.14	8.03	0.16	8.17
6	--	--	--	--	0.20	8.39	0.21	8.45	0.17	8.23	0.19	8.34
6.5	--	--	--	--	--	--	--	--	0.23	8.53	0.26	8.65
7	0.05	7.00	0.04	6.88	0.30	8.78	0.35	8.95	0.29	8.76	0.31	8.83
7.5	--	--	--	--	--	--	--	--	0.36	8.98	0.38	9.03
8	0.07	7.34	0.06	7.14	0.45	9.20	0.47	9.24	--	--	--	--
9	0.05	7.00	0.06	7.24	--	--	--	--	--	--	--	--
10	0.12	7.85	0.08	7.47	0.82	9.80	0.86	9.85	--	--	--	--
11	0.10	7.73	0.08	7.51	--	--	--	--	--	--	--	--
12	0.12	7.91	0.10	7.65	--	--	--	--	--	--	--	--
13	0.16	8.15	0.12	7.83	--	--	--	--	--	--	--	--
14	0.18	8.28	0.18	8.26	--	--	--	--	--	--	--	--
15	0.21	8.44	0.16	8.17	--	--	--	--	--	--	--	--
21	0.44	9.18	0.36	8.98	--	--	--	--	--	--	--	--

Table 40. P. cuprodurans growth curve data for 25, 30 and 35 C.

	25 C				30 C				35 C			
Time (h)	A_{420}	ln A+10	A_{420}	ln A+10,	A_{420}	ln A+10	A_{420}	ln A+10	A_{420}	ln A+10	A_{420}	ln A+10
0	0.04	6.78	0.04	6.7	0.06	7.19	0.07	7.34	0.06	7.19	0.06	7.19
3	0.10	7.79	0.10	7.79	0.12	7.87	0.13	7.95	0.13	7.96	0.13	7.96
4	0.16	8.17	0.16	8.17	0.22	8.48	0.25	8.61	0.20	8.39	0.19	8.34
5	0.28	8.72	0.27	8.69	0.43	9.16	0.49	9.29	0.32	8.86	0.29	8.76
5.5	0.40	9.08	0.40	9.08	0.60	9.49	0.66	9.58	0.39	9.06	0.34	8.92
6	0.51	9.33	0.50	9.31	0.77	9.74	0.81	9.79	0.50	9.31	0.46	9.22
6.5	0.61	9.50	0.62	9.52	0.94	9.94	0.96	9.96	0.63	9.54	0.61	9.51
7	--	--	--	--	--	--	--	--	0.72	9.67	0.69	9.63

Regression coefficients, r, ranged from 0.973 at 7 C to 0.996 at 25 C indicating that the growth data fit the growth curve well. This data is summarized in Table 41.

Table 41 and Fig. 15 demonstrated the effect of temperature on μ'. At temperatures below $T_{\mu', max}$, μ' increased linearly with temperature, then decreased rapidly to zero as incubation temperature was increased further.

The taxonomic data for P. cuprodurans were consistent with the criteria for assignment to the Pseudomonas genus (Buchanan and Gibbons, 1974). It was a gram-negative, polarly flagellated rod that produced the pigment fluorescein.

Comparison Between 1976 and 1970 Operational Taxonomic Unit

To distinguish between the P. cuprodurans culture with which I worked, and the one McCarthy (1971) described, I have adopted Sneath and Sokal's (1973) convention; the cultures were designated 1976 operational taxonomic unit (OTU) and 1971 OTU, respectively. (In so doing, I was able to delay defining the magnitude of the unit until after I had completed the comparison.) One of the limitations with which I was faced was the relatively few characteristics that McCarthy had investigated. Baumann et al. (1972) and Colwell and Wiebe (1970) recommended comparing 200-300 traits for numerical taxonomic comparisons. I compared 59 characteristics.

I summarized the results of the comparison in Table 42, scoring the comparison by giving one point for each

Table 41. Effect of temperature on P. cuprodurans growth rate, μ′.

T° C	μ	[1] n	[2] r	[3] s_{xy}
7	0.18	11	0.97	0.111
15	0.38	10	0.99	0.092
20	0.46	10	0.98	0.060
25	0.51	12	1.00	0.057
30	0.60	12	0.99	0.082
35	0.36	16	0.99	0.118
40	0.00	--	--	--

Notes:

1) n is the number of data points used to compute μ
2) r is the regression coefficient
3) s_{xy} is the standard error of the estimate

Fig. 15. Effect of temperature (C) on P. cuprodurans growth rate (μ).

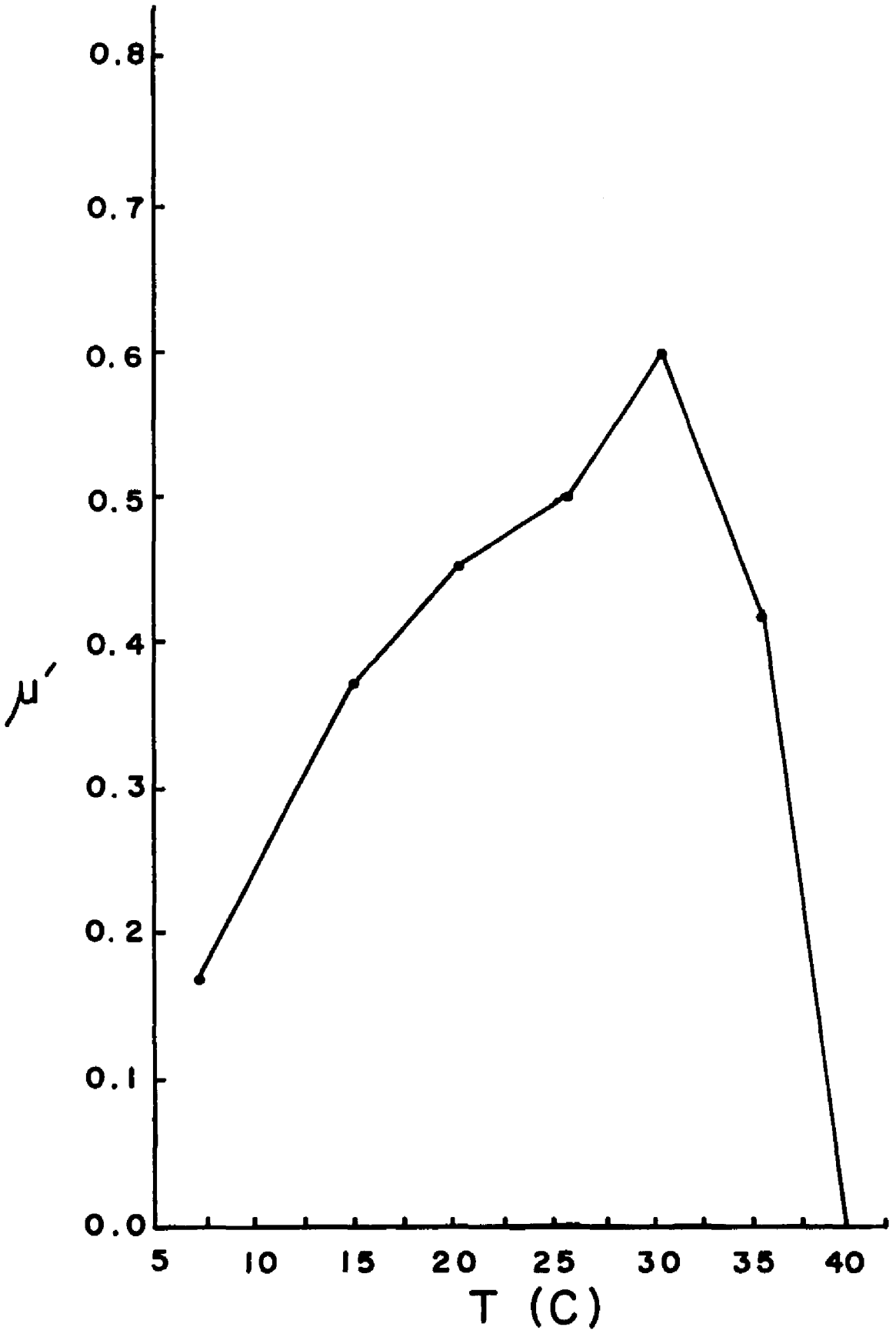

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
μ'
5
10
15
20
25
30
35
40
T (C)

Table 42. Comparison of taxonomy of P. cuprodurans (1971) and P. cuprodurans (1976).

TRAIT	[1]POSITIVE AGREEMENT	NEGATIVE AGREEMENT	DISAGREEMENT
Cell Morphology			
Shape	1	0	0
Motility	1	0	0
Flagellation	1	0	0
Grouping	1	0	0
Gram reaction	0	1	0
Endospore	0	1	0
Colony Morphology			
Form	1	0	0
Elevation	0	0	1
Margin	1	0	0
Pigmentation	1	0	0
Surface	1	0	0
Consistancy	1	0	0
Opacity	1	0	0
Agar stroke, form	1	0	0
Nutrition and Physiology			
Gelatin liquification	0	0	1
Starch hydrolysis	0	0	1
Indole production	0	1	0
Nitrate reduction	0	1	0

Table 42. continued.

TRAIT	POSITIVE AGREEMENT	NEGATIVE AGREEMENT	DISAGREEMENT
H_2S production	0	1	0
Citrate utilization	1	0	0
Catalase	0	0	1
Acetylmethylcarbanol	0	1	0
Oxidase	1	0	0
Growth in Carbohydrates (Phenol Red Broth)			
Arabinose	1	0	0
Galactose	1	0	0
Dextrin	1	0	0
Glucose	1	0	0
Lactose	1	0	0
Mannitol	1	0	0
Sucrose	1	0	0
Xylose	1	0	0
Acid Production in Carbohydrates (Phenol Red Broth)			
Arabinose	0	1	0
Dextrin	0	1	0
Galactose	0	1	0
Glucose	1	0	0
Lactose	0	1	0
Mannitol	0	1	0

Table 42. continued.

TRAIT	POSITIVE AGREEMENT	NEGATIVE AGREEMENT	DISAGREEMENT
Sucrose	1	0	0
Xylose	0	1	0
Acid Production in Carbohydrates (Synthetic Base Broth)			
Arabinose	0	0	1
Dextrin	1	0	0
Galactose	1	0	0
Glucose	1	0	0
Lactose	0	0	1
Mannitol	0	0	1
Sucrose	0	0	1
Xylose	0	0	1
Ionic Requirements			
Na	1	0	0
Ca	1	0	0
Mg	1	0	0
K	0	0	1
$SO_4^=$	0	0	1
Br^-	0	1	0
Sr^-	0	1	0
F^-	0	1	0
HCO_3^-	0	1	0

Table 42. continued.

TRAIT	POSITIVE AGREEMENT	NEGATIVE AGREEMENT	DISAGREEMENT
Temperature Limit	1	0	0
Temperature Optimum	1	0	0
Totals	a = 32	b = 16	u = 11

$$m = a + b = 48$$

$$S_{sm} = \frac{m}{m + u} = \frac{48}{57} = 0.81$$

Notes:

1) One point is assigned to appropriate column for each trait. Positive agreement exists if both OTU's share a trait; negative if neither exhibits the trait; disagreement if only one OTU exhibits the trait.

characteristic. If both OTU's demonstrated a characteristic, I listed the score under positive agreement. When neither OTU demonstrated a trait, as for indole production, I considered them to be in negative agreement. I indicated disagreement where the OTU's gave different reactions. For example OTU 1971 liquified gelatin, but OTU 1976 did not.

Comparing 59 traits, I observed disagreement for 11. OTU 1976 and OTU 1971 primarily differed in their respective abilities to hydrolyze starch and protein and produce acid from carbohydrates in synthetic base broth.

Computing the Sneath and Michner index, S_{sm} (Sneath and Sokal, 1973), I obtained the value $S_{sm} = 0.81$. On the basis of Hodgkiss and Shewan's (1968) discussion, I concluded that both OTU's belonged to the same species, but represented different strains. Since I used a culture which presumably descended from the holotype described by McCarthy, I considered the original strain to be lost. In accordance with LaPage et al. (1976), I recommended naming my strain Pseudomonas cuprodurans 1976 (proposed neotype).

Continuous Cultivation of P. cuprodurans: Calibration of Preparative and Analytical Techniques

Cultural Parameters

A standardized series of procedures for harvesting, washing, ashing, storing, and analyzing bacterial samples evolved from the preceding experiments. Continuous

cultivation offered the most suitable means of obtaining two to three g (dry weight) homogeneous bacterial samples, since physicochemical parameters and population densities could be maintained indefinitely in a dynamic steady state. Coupling continuous harvest with continuous culture eliminated the time dependence of both processes. Developing a continuous cultivation system completed the standardization process for all seven sucessive procedures. I next had to determine the overall precision for bacterial elemental analysis and the magnitude of compositional change required to permit quantification of the effect of environmental parameters on the elemental composition of a culture.

Selecting P. cuprodurans as a typical marine bacterium which was amenable to continuous cultivation, I determined the precision with which physico-chemical parameters of the cultivation system, the steady-state population densities and dry weight yields, ash yields, and elemental analyses among experiments could be reproduced.

I monitored and compared salinity (ppt), pH, temperature (C), and growth medium flow rate (ml/min) before and during five continuous culture experiments (Table 43). I did not meter the flow of filter-sterilized laboratory air or the pO_2. Salinity and pH were controlled carefully among the five experiments, and temperature varied by only 1.10 %. Flow rate varied substantially more than the other three variables reflecting the lack of precision in the adjustment of flow rate through the peristaltic pump and the absence of a metering valve downstream of the pump.

Table 43. Summary of physicochemical data for continuous cultivation of *P. cuprodurans*.

Experiment	Parameter			
	[1]S (ppt)	[2]pH	T (C)	Flow Rate (ml/min)
1	26.5	7.50	29.5	3.07
2	26.5	7.50	30.0	3.27
3	26.5	7.51	30.5	3.89
4	26.5	7.50	30.0	3.60
5	26.5	7.51	30.0	4.22
Σx	132.5	37.52	150.0	18.05
$\bar{x}$	26.5	7.50	30.0	3.61
s_x	0.00	0.005	0.35	0.463
CV_x(%)	0.00	0.07	1.10	12.8

Notes:
1) S (ppt) was assayed once, after filter sterilization.
2) pH, T, and flow rate were monitored throughout the cultivation period. Each figure represents the mean of ten observations during a CC run.

Table 44 summarized the $\bar{A}_{420}$; growth rate, μ'; and dry weight yield, Y_{dw}, for the five continuous culture experiments. Y_{dw} and μ' varied by 13.4 and 12.1 %, respectively. I analyzed the $\bar{A}_{420}$ variance among experiments (Table 45) and found that differences among experiments were significant at the 95 % confidence level. The $SS_{experiments}$ was 2.28, of this, 1.42 was due to the difference between experiment five and all other experiments. By comparison, the contributions of experiments two, three, and four were minor.

Despite precautions I had taken to minimize inter-experimental variations in growth medium composition, most of the criteria used for comparing five continuous cultivations varied by more than 10 %. I had controlled air and growth medium flow rates crudely. Growth medium flow rates during a continuous cultivation varied by 2.0 to 11.0 %. Factors contributing to this problem included clogging of the inlet orifice and the heated portion of the inlet tube. Foaming became a problem during most experiments frequently filling the entire reaction vessel free volume and clogging the vent filter.

I could only vary the pumping speed of the peristaltic pump by discrete increments. At the increment one speed slower than the one at which wash out occurred, $\bar{A}_{420}$ was no longer limited by flow rate. I demonstrated this by calculating the correlation coefficient, r, between $\bar{A}_{420}$ and mean flow rate for the five experiments. The calculated value for r was 0.54; t_s, the criterion by which I tested r

Table 44. Summary of P. cuprodurans growth and yield data from five continuous cultivations.

Experiment	$^{1}\bar{A}_{420}$	$^{2}\mu'$	$^{3}Y_{dw}$ mg/l
1	1.37	0.18	207
2	1.78	0.20	215
3	1.45	0.22	238
4	1.73	0.22	200
5	1.99	0.25	256
Σx	8.32	1.07	1,116
$\bar{x}$	1.66	0.21	223
s_x	0.253	0.026	30.0
CV_x (%)	15.2	12.1	13.4

Notes:

1) $\bar{A}_{420}$ = mean of 10 determinations

2) $\mu' = \frac{F\ (liter/h)}{V(liter)}$

3) Y_{dw} = mg (dry wt) P. cuprodurans / liter 2216E (modified)

Table 45. Analysis of variance (ANOVA) for A_{420} among continuous cultivation experiments.

a. A_{420} Data

	Experiment					
	1	2	3	4	5	
	1.07	1.48	1.49	2.05	1.60	
	1.35	1.81	1.40	1.87	1.57	
	1.09	1.82	1.40	1.89	2.00	
	1.40	1.36	1.40	1.43	2.05	
	1.70	1.58	1.42	1.26	2.16	
	1.60	2.04	1.29	0.94	2.07	
	1.20	1.75	1.34	1.73	2.07	
	1.10	1.97	1.43	1.59	1.68	
	1.18	1.93	1.42	1.59	2.06	
	1.90	1.58	1.57	1.59	2.20	
Σx_j	13.59	17.32	14.16	15.94	19.46	$\Sigma\Sigma x_j$ = 80.47
$\bar{x}_j$	1.36	1.73	1.41	1.59	1.94	$\bar{G}$ = 1.60
$(\Sigma x_j)^2$	134.69	299.98	200.50	254.08	378.69	$\Sigma(\Sigma x_j)^2$ = 1317.89
Σx_j^2	19.22	30.45	20.10	26.36	38.36	$\Sigma x^2 j$ = 139.49

b) ANOVA Summary

Source of Variation	SS	df	MS	F
Experiment	2.28	4	0.57	9.5
Error	2.70	45	0.06	
Total	4.98	49		

$F_{.95}(4,45) = 2.61$

c) Comparison Among Treatment Means [1]

Experiment	SS
1	0.78
2	0.18
3	0.31
4	0.003
5	1.42

Note:

1) Comparison is SS due to difference between experiment indicated and all other experiments.

for significance, was 2.06. $T_{crit\ (.95)} = 2.35$, implying that $\bar{A}_{420}$ was not correlated to flow rate.

I also compared $\bar{A}_{420}$ with Y_{dw} and obtained values: $r = 0.41$, $t_s = 1.90$. Y_{dw} was not correlated to $\bar{A}_{420}$ at the 95 % confidence level. I attributed this to differences in the efficiency of the harvest process among experiments.

Ash Yields

Having established that there were discernible differences among the five continous cultivation experiments, I determined whether ash yield and elemental data reflected the differences. The ash and elemental data also served as indicators of wash procedure efficiency, high ash yields and major element concentrations suggesting the presence of intercellular salts and growth medium retention.

I summarized the dry ash yield data in Table 46. The mean ash yield from 50 subsamples was 7.99 ± 0.483 %. Analysis of variance supported acceptance of the alternative hypothesis which stated that difference among ash yields from different experiments was significant. The variation due to differences among experiments, measured as SS_{exp}, was 9.02. Of this, 8.52 was due to the uniqueness of experiment three, as compared with the other four experiments. Ash yields from experiment three were higher than from any other experiment.

To determine whether mean ash yields varied with bacterial population density or dry weight yield, I plotted $\bar{A}_{420}$ and Y_{dw} as functions of ash yield (Fig. 16). Had a

Table 46. ANOVA for ash yields among five continuous cultivation experiments.

a. Ash Yield Data

	Experiment				
	1	2	3	4	5
[1]	8.09	7.30	8.65	8.08	7.80
	7.68	7.08	8.77	7.90	7.72
	7.54	7.79	8.95	7.84	7.61
	6.93	7.55	8.85	7.91	8.24
	7.67	7.88	8.98	7.86	7.87
	7.54	8.14	8.77	7.81	7.74
	7.71	7.58	8.86	7.91	7.98
	7.79	7.77	8.80	7.98	7.77
	7.75	8.15	8.70	7.80	7.90
	7.52	8.21	8.81	7.99	7.98
Σx_j	76.22	77.36	88.14	79.08	78.61
					$G = \Sigma\Sigma x = 399.41$
$\bar{x}_j$	7.62	7.74	8.81	7.91	7.86
					$\bar{G} = 7.99$
SS_j	0.78	1.18	0.09	0.07	0.29
					$SS_j = 2.41$

b. ANOVA Summary

Source of Variation	SS	df	MS	F
Experiment	9.02	4	2.26	41.85
Error	2.41	45	0.054	
Total	11.43	49		$f_{.95(4,45)} = 2.61$

c. [2]Comparison of Means

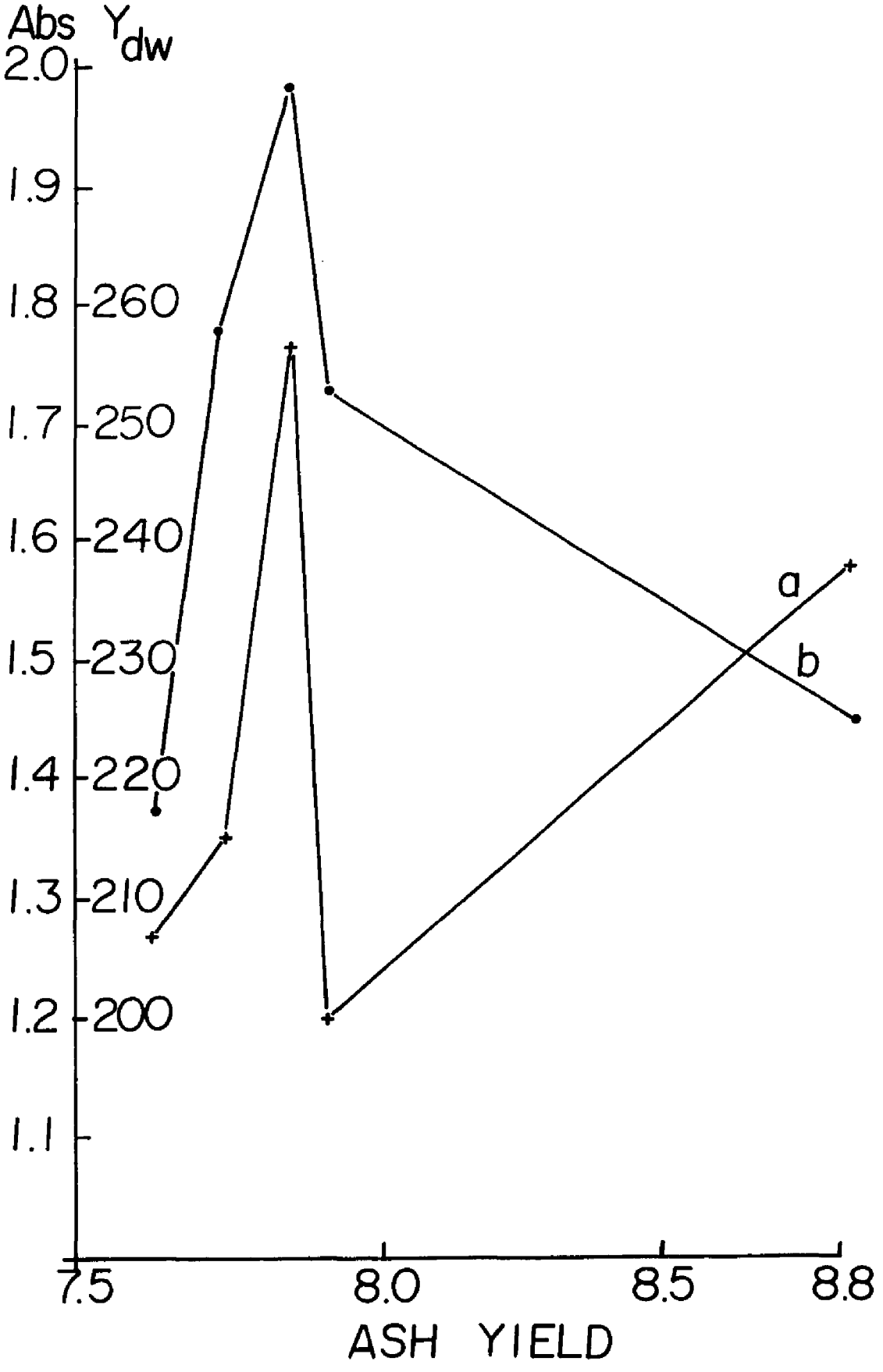
$SS_{experiment}$[3] = 3.52

Notes:

1) Figures are (Ash wt/Dry wt) x 100

2) $SS_{run\ 3}$ is due to difference between experiment 3 and other four experiments

Fig. 16. $\bar{A}_{420}$ and Y_{dw} vs. ash yield (% dry weight), for P. cuprodurans grown in continuous cultivation apparatus. Curve a) Y_{dw}(mg dw/liter); curve b) $A_{420\ nm}$.


Abs Y_{dw}
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
260
250
240
230
220
210
200
a
b
7.5
8.0
8.5
8.8
ASH YIELD

correlation existed, I would have obtained a linear or curvilinear plot. Neither function produced such a curve. Therefore, I concluded that ash yield variation among experiments was not related to population density or dry weight yield.

A third possibility, one which I discussed relevant to ash technique optimization, was that ash yield differences were due to variations in the combustion procedure. I discounted this, since I had dry ashed subsamples from experiments three and four together.

Since the overall coefficient of variation for ash yields was 6.04 %, I concluded that the precision of the ashing procedure was acceptable. The variation observed was minor and I will address it again once I have presented the elemental data.

Elemental Composition

Concentrations of the major protoplasmic elements provided a second criterion for evaluating the efficiency of the wash technique. Variation introduced while preparing samples for analysis by atomic absorption spectroscopy was absent since I performed C, H, N, and S analyses on the ground, dry sample.

As demonstrated in Table 47, coefficients of variation for C, H, N, and S were 1.95, 2.65, 1.46, and 2.40 % respectively. The C, H, N concentrations were much higher than those obtained during preliminary studies. The reasons for this were two-fold. I had harvested the bacteria

Table 47. Precision of C, H, N and S analyses for *P. cuprodurans* among five continuous cultivation experiments.

Experiment	% Dry Weight Elemental Composition			
	C	H	N	S
1	52.8	7.53	14.4	---
	53.8	7.47	14.6	---
2	52.6	7.47	14.6	0.54
	51.9	7.56	14.3	---
3	50.5	7.47	14.0	0.52
	50.7	7.09	14.1	---
4	51.6	7.50	14.5	---
	51.5	7.18	14.3	---
5	52.6	7.30	14.3	0.51
	52.6	7.03	14.1	---
$\bar{x}$	52.1	7.36	14.3	0.52
S_x	1.02	0.195	0.21	0.012
C.V. %	1.95	2.65	1.46	2.40

unwashed or washed them only once during the preliminary investigation. Low C, H, N accompanied by high Na, K, Ca and Mg concentrations clearly indicated the presence of intercellular contamination. The second factor was in adjusting the operating parameters of the C, H, N analyzer. Since the high Na, K, Ca and Mg concentrations could not account for all of the difference between theoretical and observed C, H, N concentrations, I hypothesized that a matrix effect was involved. Having carefully pulverized the samples, I proceeded with the C, H, N analyses, using 30 sec combustions. Variation among analyses was substantial, and C, H, N values remained low. By increasing the combustion interval to 60 sec, I was able to eliminate the matrix effect. The problem had been incomplete sample combustion.

The precision of C, H, N and S concentrations among the five experiments abrogated the hypothesis that interexperimental ash yield variation was due to inconsistencies in the wash procedure. I further substantiated this by subsequent elemental analyses.

Unable to determine O directly, I calculated the O content to be approximately 17.7 % of the dry weight by subtraction. I assayed P colorimetrically as total PO_4^{-3}. Variation among subsamples within an experiment ranged from 8.65 to 26.7 % (Table 48). Variation among experiments was significant at the 95 % confidence level. I found no correlation between P concentration and the cultural criteria or ash yield.

Table 48. P content of P. cuprodurans from five continuous cultivation experiments.

a. (µgP/µg DW) x 100 and Elementary Statistics

	Experiment				
	1	2	3	4	5
[1]	2.22	1.86	1.43	1.16	1.71
	1.79	1.87	1.96	0.91	2.61
	2.09	1.86	1.23	1.03	1.49
	1.91	1.71	1.97	1.28	2.17
	2.28	2.13	2.67	0.98	2.03
	2.29	1.73	1.45	0.96	2.31
	2.09	2.06	2.29	0.93	2.12
	1.48	1.77	1.78	1.23	1.98
	2.22	1.62	1.17	1.30	2.10
	2.15	1.98	1.87	1.02	1.89
Σx	20.52	18.58	17.82	10.80	19.96
$\bar{x}$	2.05	1.86	1.78	1.08	2.00
S_x	0.257	0.161	0.475	0.149	0.243
CV_x %	12.5	8.65	26.7	13.8	12.2

b. ANOVA Summary

Source of Variation	SS	df	MS	[2] F
Experiments	6.09	4	1.52	19.0
Error	3.58	45	0.08	
Total	9.67	49		

Notes:

1) Each figure represents mean of triplicate assay

2) $F_{.95\ (4,45)} = 2.61$

Coefficients of variation for Na concentrations ranged from 13.4 to 37.2 % (Table 49). For K, intra-experimental variation was 9.10 to 26.3 % (Table 50). Ca varied from 11.8 to 29.5 % within different experiments (Table 51), and Mg varied from 12.6 to 35.9 % (Table 52). Variations for Fe (Table 53), Zn (Table 54), and Cu (Table 55) in subsamples were 9.58 to 35.2 %, 12.9 to 27.4 % and 11.4 to 28.7 %, respectively. Variation among experiments was significant for Na, K, Ca, Mg, Fe, Zn, and Cu.

To determine whether intra-experimental variations for difference elements were systematic, I graphed the concentration trends for each element (Fig. 17). Similarities in the response of different elements to experimental treatments would result in similar curves. Since I was comparing trends, the scales selected for ordinates were not necessarily identical for each element. Trends for P, Mg, Fe, Zn, and Cu were quite similar, while those for K, Na and Ca were less so. Graphs for the former five elements indicated that concentrations in P. cuprodurans from the second, third and fourth experiments decreased relative to the first experiment. Concentrations of all elements except Cu were minimal in subsamples from experiment four, and most concentrations were maximal in subsamples from experiment five. To a certain extent, these trends were similar to that for Y_{dw} (Table 42). The Y_{dw} pattern for experiments three, four and five would suggest that elemental composition was correlated to Y_{dw}. However, except for Ca, the comparison did not remain consistent for

Table 49. Na content of P. cuprodurans from five continuous cultivation experiments.

a. [1]PPM Na and Elementary Statistics

	Experiments				
	1	2	3	4	5
	2510	1580	2460	2510	4420
	2060	1920	3620	1760	4600
	2740	2150	2110	1920	3480
	2930	1330	3700	2210	4650
	2840	2240	1600	1700	6540
	3100	1680	2690	1640	5280
	2460	2220	968	1740	4240
	1860	1610	3300	2170	4160
	2470	2230	2080	2160	4580
	2670	2050	3970	1800	3890
Σx	25610	19830	26550	19610	45840
$\bar{x}$	2560	1980	2660	1960	4580
s_x	381	267	988	286	839
CV_x%	14.9	13.4	37.2	14.6	18.3

b. ANOVA Summary

Source of Variation	SS	df	MS	[2]F
Experiments	4.61×10^7	4	1.15×10^7	28.9
Error	1.79×10^7	45	3.89×10^5	
Total	6.40×10^7	49		

Notes:

1) PPM Na as fraction of dry weight

2) $F_{.95\ (4,45)} = 2.61$

Table 50. K content of P. cuprodurans from five continuous cultivation experiments.

a. [1]PPM K and Elementary Statistics

	Experiments				
	1	2	3	4	5
	2660	1500	1740	2090	2900
	2450	3030	1870	1300	3110
	2690	2920	1850	1720	2700
	2550	3130	1860	2230	3130
	2690	3020	1260	1530	3080
	2780	3010	1720	1770	3250
	2850	3230	1860	1340	3110
	2080	2880	1880	1790	3010
	2620	2170	1260	1970	2940
	2940	3100	2760	1530	2890
Σx	26310	27990	18060	17270	18080
$\bar{x}$	2630	2800	1810	1730	1810
S_x	240.	541	413	309	476
CV_x%	9.10	19.3	22.9	17.9	26.3

b. ANOVA Summary

Source of Variation	SS	df	MS	[2]F
Experiments	1.07×10^7	4	2.68×10^6	15.95
Error	7.58×10^6	45	1.68×10^5	
Total	2.46×10^7	49		

Notes:

1) PPM K as fraction of dry weight

2) $F_{.95(4,45)} = 2.61$

Table 51. Ca content of P. cuprodurans from five continuous cultivation experiments.

a. [1]PPM Ca and Elementary Statistics

	Experiments				
	1	2	3	4	5
	2300	1400	3090	3020	2160
	1800	3420	3610	1780	2470
	2400	3300	3390	2400	2160
	2200	3500	3500	2540	2450
	2700	3900	2100	1300	2560
	2560	3010	3370	1820	3060
	2090	3640	3640	1190	2670
	1580	2880	3130	1960	2600
	2020	2380	1780	2060	2590
	2390	3270	3390	1470	2080
Σx	22040	30700	31000	19540	24800
$\bar{x}$	2200	3070	3100	1950	2480
S_x	342	724	641	576	293
CV_x%	15.5	23.6	20.7	29.5	11.8

b. ANOVA Summary

Source of Variation	SS	df	MS	[2]F
Experiments	3.06×10^8	4	7.64×10^7	261
Error	1.32×10^7	45	2.93×10^5	
Total	3.19×10^8	49		

Notes:

1) PPM Ca as fraction dry weight

2) $F_{.95(4,45)} = 2.61$

Table 52. Mg content of P. cuprodurans from five continuous cultivation experiments.

a. [1]PPM Mg and Elementary Statistics

	Experiments				
	1	2	3	4	5
	5430	2100	2630	2460	4500
	4470	4560	4350	1730	5800
	4920	4620	1690	2170	3450
	4720	4190	4160	2350	5650
	5710	5410	1620	1920	5280
	5510	4160	3690	1880	5680
	4950	4880	5020	1880	5220
	3290	4430	3660	2490	4960
	5390	4470	2210	2320	5060
	5330	5050	4080	2120	4720
Σx	49720	43870	33110	21320	50320
$\bar{x}$	4970	4390	3310	2130	5030
S_x	707	891	1187	270	697
CV_x%	14.2	20.3	35.9	12.6	13.8

b. ANOVA Summary

Source of Variation	SS	df	MS	[2]F
Experiments	6.10×10^7	4	1.36×10^7	24.8
Error	2.46×10^7	45	5.47×10^5	
Total	8.56×10^7	49		

Notes:

1) PPM Mg as fraction of dry weight

2) $F_{.95(4,45)} = 2.61$

Table 53. Fe content of P. cuprodurans from five continuous cultivation experiments.

a. [1]PPM Fe and Elementary Statistics

	Experiments				
	1	2	3	4	5
	162	131	147	144	204
	129	177	163	82.0	197
	176	154	101	116	181
	145	168	130	148	203
	218	224	74.8	122	252
	286	177	146	134	239
	235	177	135	137	207
	220	134	137	118	216
	181	362	110	130	220
	173	243	119	104	218
Σx	1925	1947	1263	1235	2137
$\bar{x}$	192	195	126	124	214
S_x	47.1	68.5	25.8	19.8	20.5
CV_x%	24.5	35.2	20.4	16.1	9.58

b. ANOVA Summary

Source of Variation	SS	df	MS	[2]F
Experiments	7.80×10^4	4	1.95×10^4	11.7
Error	7.50×10^4	45	1.67×10^3	
Total	15.30×10^3	49		

Notes:

1) PPM Fe as fraction of dry weight

2) $F_{.95(4,45)} = 2.61$

Table 54. Zn content of P. cuprodurans from five continuous cultivation experiments.

a. [1]PPM Zn and Elementary Statistics

	Experiments				
	1	2	3	4	5
	86.3	135	72.6	76.3	92.8
	89.4	91.5	85.2	43.5	101
	114	123	95.8	69.9	111
	113	108	85.8	81.5	95.0
	116	128	30.9	35.6	98.7
	195	126	66.8	70.4	97.1
	130	126	68.7	46.5	99.8
	183	89.4	85.5	68.0	131
	137	79.7	55.3	74.7	88.7
	139	150	77.6	58.3	121
Σx	1303	1157	724.2	724.7	1036
$\bar{x}$	130	116	72.4	62.5	104
S_x	35.7	22.6	18.71	15.66	13.4
CV_x%	27.4	19.5	25.8	25.1	12.9

b. ANOVA Summary

Source of Variation	SS	df	MS	[2]F
Experiments	3.31×10^4	4	8275	16.2
Error	2.30×10^4	45	511	
Total	5.61×10^4	49		

Notes:

1) PPM Zn as fraction of dry weight

2) $F_{.95(4,45)} = 2.61$

Table 55. Cu content of P. cuprodurans from five continuous cultivation experiments.

a. [1]PPM Cu and Elementary Statistics

	Experiments				
	1	2	3	4	5
	31.8	24.2	24.8	19.1	44.0
	22.7	24.6	30.6	37.0	34.6
	35.9	25.3	36.5	21.1	35.6
	34.6	29.9	16.1	23.5	29.7
	24.7	23.0	26.8	30.0	34.3
	24.2	22.0	20.4	12.9	33.3
	23.2	31.2	23.9	31.2	34.8
	18.1	30.8	13.3	26.6	32.7
	29.7	27.3	22.4	25.2	40.6
Σx	275.2	254.6	235.2	247.9	356.8
$\bar{x}$	27.5	25.5	23.5	24.8	35.7
S_x	5.78	4.58	6.76	6.85	4.08
$CV_x\%$	21.0	18.0	28.7	27.6	11.4

b. ANOVA Summary

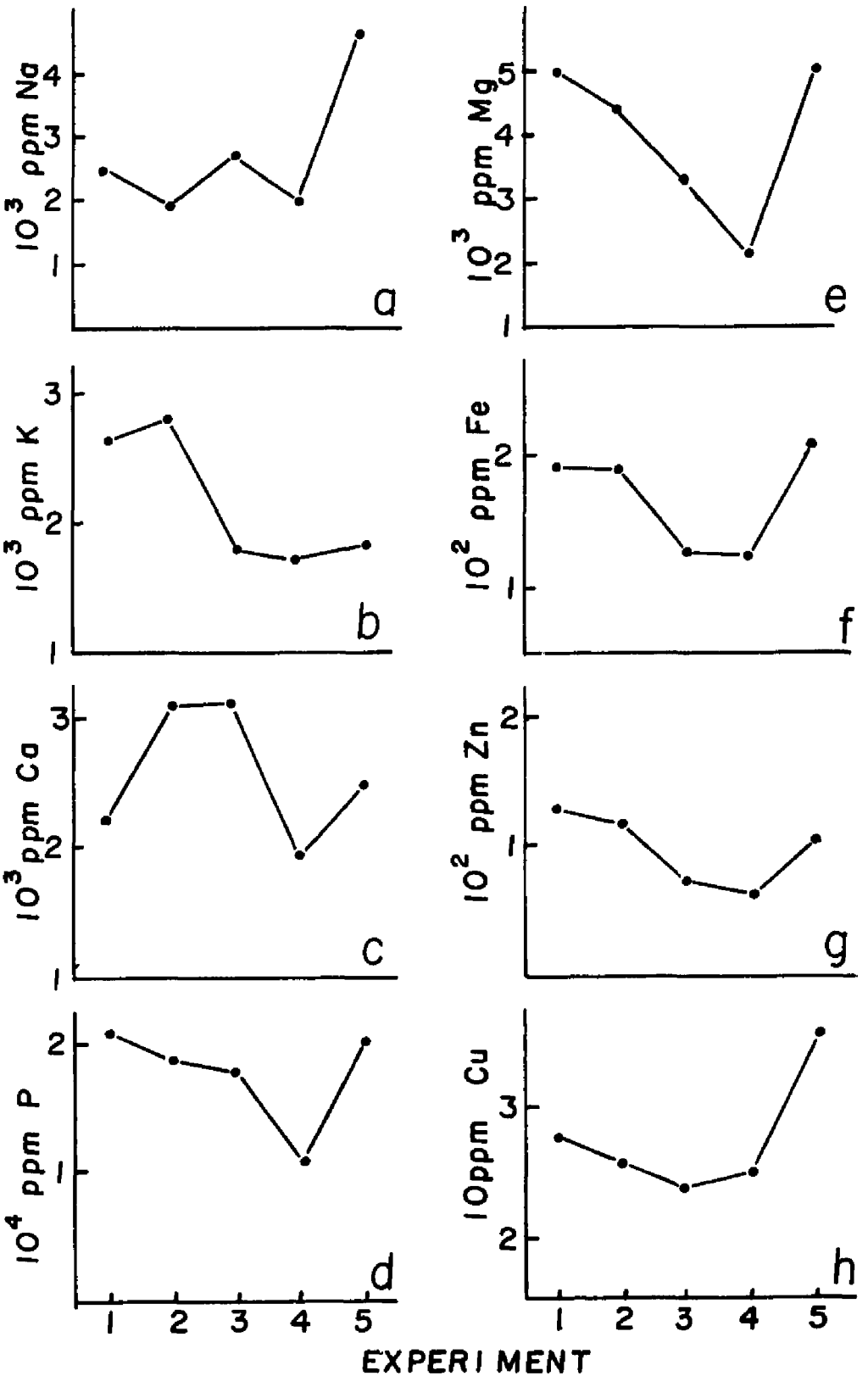
Source of Variation	SS	df	MS	[2]F
Experiments	940	4	235	7.19
Error	1470	45	32.7	
Total	2410	49		

Notes:

1) PPM Cu as fraction of dry weight

2) $F_{.95(4,45)} = 2.61$

Fig. 17. Comparative trends of elemental concentration means, as functions of continuous cultivation experiments. Numbers on the ordinate represent first significant digit for concentration. a) Na, 0 to 5 x 10^3 ppm; b) K, 1 to 3 x 10^3 ppm; c) Ca, 1 to 3 x 10^3 ppm; d) P, 0 to 2 x 10^4 ppm; e) Mg, 1 to 5 x 10^3 ppm; f) Fe, 0.5 to 2 x 10^2 ppm; g) Zn, 0 to 2 x 10^2 ppm; h) Cu, 1.5 to 3 x 10^1 ppm. All concentrations are fractions of dry weight.


10^3 ppm Na
a
10^3 ppm Mg
e
10^3 ppm K
b
10^2 ppm Fe
f
10^3 ppm Ca
c
10^2 ppm Zn
g
10^4 ppm P
d
10ppm Cu
h
1 2 3 4 5
EXPERIMENT

experiments one and two. Although the data did not conclusively substantiate a correlation between Y_{dw} and elemental composition, it did suggest that factors which affected Y_{dw} also affected the mineral content of P. cuprodurans. This phenomenon should be given careful scrutiny.

The cultural, ash and elemental data indicated that within an experiment, data was sufficiently precise to resolve minor differences among continuous cultivation experiments. This suggested that the contributions of ash treatment, analate preparation, and assay to experimental error were not significant compared with the cultivation, harvest and wash procedures. Since most of the elements analyzed showed similar concentration trends among experiments, I concluded that the wash procedure was the variable which contributed most significantly to the total variation. This was supported by the asystematic pattern for elemental concentrations among experiments and the lack of correlation between Y_{dw} and $\bar{A}_{420}$.

Given the limitations imposed determining the elemental composition of a bacterium grown in a defined system, I calculated the number of repeated observations necessary to be 80 % certain of resolving a 10 % or 20 % concentration difference for a particular element at the 95 % confidence level (Table 56). The iterative computation procedure was from Sokal and Rohlf (1969) and was based upon the observed average coefficient of variation for each element among the five experiments. To resolve 10 % differences in P concentrations, I would need 30 subsamples

Table 56. Number of replications needed to detect 10% and 20% concentration differences between means.

Element	[1]CV%	Number of Samples Needed [2]True Difference to be Detected	
		10%	20%
P	14.8	30	10
Na	19.7	50	15
K	19.1	50	15
Ca	20.1	55	15
Mg	19.4	50	15
Fe	21.2	60	15
Zn	22.1	65	20
Cu	21.3	60	15

Notes:

1) $\overline{CV}$% average CV among five experiments
2) Number of samples were rounded off to next higher five. Example: if 11.2 samples was calculated, figure rounded off to 15.

from each experiment. For Zn, I would require 65 subsamples. Using 150 mg subsamples, Zn concentrations in the analate were close to the detection limits. I would have to harvest approximately 10 g dry bacteria which equals to 50 liters of medium from each experiment. I could resolve 20 % differences among means between experiments using 15 subsamples for all elements except Zn which would require 20.

From these calculations, I concluded that with a few refinements to the continuous cultivation aparatus, the methodology developed would provide a satisfactory means for determining the effects of various physio-chemical parameters on the elemental composition of bacteria.

The number of elements for which I could perform analyses was limited by the sample volume and the concentrations of elements in the analate. I could circumvent these problems by using a graphite furnace rather than a flame for atomizing the sample. The furnace required 2 µl aliquants, as compared with 7 - 10 ml/min nebulized into the flame. Using small volumes would permit use of substantially smaller volumes into which I would redissolve bacterial ash. The net effect would be to increase the concentration of elements in the analate by five-fold or more. However, there are limitations to flameless atomic absorption spectroscopy which have been mentioned in the literature review and which I shall consider in the discussion.

I concluded that the elements present at $\geq$ 10 ppm provided a sufficient basis for investigating compositional variations induced by environmental changes. In light of the

limitations of flame atomic absorption spectroscopy, resolution of trace element variations should be resolved only after models have been developed to describe the relationships between major elements and physico-chemical parameters.

I summarized the composition of P. cuprodurans from the five continuous cultivation experiments in Table 57. Na, K, Ca and Mg were all present in approximately the same concentrations. I distinguished three groups of elements in the ash by their concentrations. P alone was present at greater than 10^4 ppm. Na, K, Ca, Mg were clustered around 2.5×10^3 ppm; and Fe, Zn and Cu were clustered at the lower end of $\geq 10^2$ range. A fourth group of metals, including Mn, Cd, Ni, and Pb were present in concentrations < 10 ppm. On a molal basis, Mg was the most predominant cation.

Eight elements were assayed in the ash. In total, they accounted for 35.3 % of the ash. Suspecting that other elements besides O for which analyses had not been performed, were present at significant concentrations, I had representative subsamples analyzed by two additional techniques. Dr. David Swift of the Central Industrial Instrumentation Division, University of New Hampshire, performed optical emission spectrography on a sample of dry ashed P. cuprodurans. Dr. Frank DiMiglio and Dr. Michael Doyle, Director and Assistant Director of the Rhode Island Nuclear Science Center, Naragansett, assisted me in performing neutron activation analysis on P. cuprodurans in various states and in various

Table 57. Elemental composition of P. cuprodurans grown in filter-sterilized modified 2216E broth medium at pH 7.51, 30 C.

Element	[1]PPM ± s	[2]CV %	μ mole/μg DW
C	541000 ± 10200	1.95	43400
N	143000 ± 2100	1.46	10200
H	73600 ± 1950	2.65	73600
S	5200 ± 124	2.40	162
P	17500 ± 1980	11.3	565
Mg	4000 ± 1320	33.3	163
Na	2800 ± 1140	41.6	120
Ca	2600 ± 2550	99.6	64
K	2150 ± 780	32.9	55.0
Fe	170. ± 55.9	32.9	3.05
Zn	97. ± 33.8	34.9	1.5
Cu	27.4± 7.01	25.6	0.431

Notes:

1) $\overline{PPM} \pm s$: mean ppm as fraction dry weight for 50 ashed subsamples

2) CV % calculated as $(\sqrt{(SS_t/df_t)} / \bar{G}) \times 100$, where $\bar{G}$ is the overall mean

matrices. The semiquantitative data from the emission spectrograph and neutron activation analysis of P. cuprodurans ash were summarized in Table 58.

For most elements, I observed close agreement between the two techniques. Neutron activation analysis provided a high estimate of Mn and Cu concentrations compared with emission or atomic absorption data (Table 57), and provided low estimates of Na, Ca, and Mg concentrations. I detected two elements by neutron activation analysis, which I was unable to detect by flame atomic absorption spectrophotometry. These were Al (∿28 ppm) and V (∿7.3 ppm). To resolve other elements, I would have to develop a post-irradiation chemical separation procedure to remove abundant radionuclides such as ^{24}Na which produced several photopeaks and prevented resolution of less abundant radionuclides. I had hoped that neutron activation analysis would have obviated the sample digestion and concentration steps. However, to obtain semi-quantitative or quantitative data, I had to follow the procedure I used for preparing samples for atomic absorption analysis. I concluded that unless Al and V were of particular interest, routine neutron activation analysis did not offer any advantages over flame atomic absorption spectrophotometry.

Optical emission spectrography appeared to be a more promising technique than neutron activation analysis. I detected 14 elements on a single spectrograph. The analysis was preformed on a dry ashed sample, eliminating the problem I encountered trying to dissolve the ash residue in HNO_3 or HCl. Using a precision densiometer, one could quantitatively resolve

Table 58. Qualitative determination of 26 elements in P. cuprodurans ash by optical emission spectrography and neutron activation analysis.

Element	Approximate Concentration (ppm dry weight) Detected	
	[1]Arc-Emission	[2]Neutron Activation
Na	> 100	∿ 560
Ca	> 100	∿ 540
Mg	> 100	∿ 810
Si	> 100	[3]ND
Fe	> 100	ND
Cr	∿ 20	ND
Al	∿ 20	28
P	> 1000	ND
Mn	60-70	∿ 870
Ni	∿ 10	ND
Cu	10-20	∿ 100
Se	∿ 100	ND
Mo	∿ 10	ND
Aq	1-10	ND
K	ND	∿ 1200
V	< 5	∿ 7.3
B	< 50	ND
Ba	< 5	ND
Sr	< 500	ND
Pb	< 75	ND
As	< 100	ND
Bi	< 5	ND
Zn	< 10	ND
Cd	< 20	ND
Sn	< 10	ND
Sb	< 10	ND

Notes:

1) From spectrograph of 60 sec arc at 9.5 amps.
2) Samples were irradiated 5 min at 4 x 10^{12} neutrons/cm^2/sec flux.

Table 58. continued.

3) ND: not detected

63 elements from an optical emission x-ray spectrograph (Cowgill and Burns, 1975). The semi-quantitative data from the spectrograph (Table 58) was comparable to that from the atomic absorption spectrophotometer (Table 57). These observations suggest that emission spectrography would be a means of extending the number of elements analyzed and checking the atomic absorption data.

DISCUSSION

Errors introduced during seven sequential processes involved in culturing, harvesting, washing, drying, ashing, preparing, and analyzing organisms have limited the comparability of elemental data among different investigators. Although researchers have recommended approaches for minimizing contamination while sampling (Robertson, 1968; Grant, 1969) and for improving analytical accuracy and precision (Bowen, 1965; Brewer and Spencer, 1970), they have paid little attention to problems encountered at other stages of the process. Carpenter (1964) and Chapman (1964) addressed the problem of removing dust from foliar samples before subsequent treatment. Bryan and Hummerstone (1973) suggested rinsing algae in seawater to remove particulate contaminants; but for the most part, no earnest attempt has been made to separate organisms from contaminating menstruum carried over during harvest (for example Martin and Knauer, 1973).

Another issue which investigators working with multi-cellular organisms often acknowledged as a significant parameter was tissue differentiation. Riley and Segar (1970) demonstrated that trace element concentrations differed among tissues from specific echinoderms and coelenterates. Bryan and Hummerstone (1973) noted compositional differences along Fucus vesiculosis thalli. I recommend that bacteria be used to develop fundamental models describing the interaction

between the physico-chemical environment and the chemical composition of biological systems. However, unless parameters related to the cultivation, preparation and analysis of bacteria were defined, rigorously, investigators would be unable to compare elemental data obtained in different laboratories.

To obviate the problem, I have considered sources of variation contributed by each of the seven sequential processes and have recommended a standard procedure for each process. Before commencing the first process, media preparation, I was confronted with the problem of trace metal contamination from laboratory air, reagents and labware.

Hamilton et al. (1972) listed the predominant contaminants in laboratory air and dust. At Jackson Estuarine Laboratory, seawater aerosols produced by flowing seawater in the wet laboratory contributed to the air problem. To reduce this problem, I used a laminar flow hood when possible, and aerated cultures with filter-sterilized air. This was not as satisfactory as working in a clean-room environment.

I summarized the most common labware contaminants in Table 59. Robertson (1968) and Struempler (1973) noted that even acid-cleaned labware contributed trace metals. Once the labware had been cleaned several times, continued leeching of minerals from the labware was minimal. Robertson (1968) also reported that most attempts to purify chemicals beyond reagent grade resulted in the exchange of one contaminating

Table 59. Contamination from common labware materials[1].

Material	Extent of Contamination	Elements Like to be Involved
Stainless Steel	Often Extensive	Co, Cr, Fe, Mn, Mo, Nb, Ni, V, W, Ta
Aluminum	Moderate	Al, Cu, Mg, Mn, Na, Sc
Pyrex Glass	Moderate	Al, B, K, Na, Si, Fe
Silica	Small	Al, Cl, Ti, Zn, Fe
Polytetrafluoroethylene	Very Small	F
Kimwipe Tissue	Moderate	Zn, Fe
Millipore Filter	Small	Zn, Cr, Fe

Notes:

1) From Bowen (1966) and Robertson (1968)

element for another. By using reagents from a single lot for each series of experiments, I eliminated reagent composition as a source of interexperimental variation but not as a source of trace metal contamination.

The elemental composition of the bacteria was affected by the medium (Dawson, 1919; Jones et al., 1976c). Since modified 2216E medium supported the growth of a wide variety of bacteria (Oppenheimer and Zobell, 1952; Buck, 1974) and its composition quantitatively and qualitatively reflected the nutrient composition of at least one marine ecological niche (Sieburth et al., 1976), I used it in my investigation.

Jones (1967b) observed that 10.3 mg precipitate/liter formed when synthetic seawater at pH 7.7 was autoclaved at 121 C for 15 min. The precipitate consisted of O (46 $\pm$ 4 %), Cl (8.9 $\pm$ 0.4 %), Na (5.9 $\pm$ 0.2 %), Br (1.3 $\pm$ 0.1 %), F (0.2 $\pm$ 0.1 %), Mn (0.0142 $\pm$ 0.0007 %), Mg ($\sim$4 %), Si ($\sim$4 %) and Ca ($\sim$2 %). Jones (1967a) also observed that autoclaving seawater growth medium affected the growth of E. coli by removing potentially toxic heavy metals from solution as coprecipates with $CaCO_3$. My electrochemical and EDAX data (Table 15, Fig. 5 and 6) substantiated Jones' observations. Ramamoorthy and Kushner (1975a) observed that of 20 ppm Hg, Pb or Cu added to a complex growth medium, only $\leq$ 80 ppb remained as free cations in solution. The balance of added trace ions were complexed by the organic fraction of the growth medium. I considered these observations sufficient justification for using only

filter-sterilized growth media in trace element investigations.

As mentioned above, tissue differentiation was perhaps the most severe limitation to using multi-cellular organisms for developing models to relate environmental conditions with the organism's elemental composition. Cultivating bacteria in batch culture did not really eliminate this problem since a bacterium's composition varied with culture age (Kung et al., 1976). Harvesting cells after a predetermined time in culture had a number of disadvantages in common with harvesting cells at a particular stage in the growth curve. Unless I had harvested the cells simultaneously, I would not have obtained a completely homogeneous sample. I needed approximately a five-liter culture to obtain 1.0 g dry cell material, and had no harvesting apparatus with which I could effect a harvest quickly enough to prevent time from becoming a significant variable. Moreover, cultivating bacteria under different physico-chemical conditions would affect their growth rates meaning that either duration of exposure to the growth conditions or stage of growth cycle at harvest would vary among experiments.

By using a continuous cultivation system, I obviated the time and growth curve dependencies. Jannasch (1965, 1969) discussed this genre of chemostat application. Having established a steady-state under defined conditions, I could continually cultivate bacteria indefinitely to obtain as much cell material as needed (Ricica, 1966). Moreover, I could alter the growth conditions, wait for the population to return to a steady state and continue harvesting (Fencl, 1966).

I evaluated three methods for continually harvesting the bacteria. Two of the techniques involved filtration. The Amicon thin-channel ultrafiltration system (Amicon Technical Publication 436) would not harvest large concentrations of bacteria and separate them from the growth medium at the rate required due to membrane clogging and flow rate restriction. Sieburth (personal communication) has designed and used a large surface area, reverse flow concentrator, fabricated from plexiglass and Nuclepore membrane sheeting. Clogging, a problem with the Amicon system, was avoided since the membrane was positioned above the inlet and organisms remained in the free volume beneath the filter. Both systems shared the problem of continued bacterium-medium interaction after cells had presumably been harvested. The harvesting apparatus would have become a second stage reaction vessel.

To overcome this problem, I decided to use a continuous centrifugation system. As bacteria became part of the pellet, they were separated effectively from the growth medium. I maintained the centrifuge chamber at freezing temperatures, thereby minimizing any continued growth. At the end of harvest, cells were already in a tight pack, eliminating a potential requirement for a post-harvest concentration step. While centrifuging, I did not have to worry about back pressure building up and inhibiting the outflow of medium and cells from the reaction vessel. Thus, I found the continuous centrifugation system compatible with the continuous culture system.

The next problem was separating cells from the menstruum. How was I to obtain intact bacteria isolated from intracellular salts and growth medium? I had intended to harvest bacteria by filtration, then displace the growth medium with wash reagent just before concentrating the cell pack. The impracticability of the filtration harvest procedure eliminated this wash procedure. Hurwitz, Braun, and Peabody (1965) developed a washing procedure involving centrifuging cells through an immiscible solvent containing a mixture of two silicones. Intercellular contaminants were concentrated in the silicone layer while bacteria remained in an aqueous suspension passing through the silicone layer and formed a pellet. This technique would have contaminated the bacteria with Si rendering it unsuitable for analyses including Si.

Jones et al. (1977a) reported that after three washes in either distilled water, 0.5 N ammonium formate or 0.01 M phosphate buffer, the trace element content of A. marinus remained stable. They decided that 0.5 N ammonium formate was the most appropriate wash reagent since it volatilized during drying at 105 - 110 C (Riley and Roth, 1971), even through K decreased from 13,820 to 81 ppm after three washes. I found that N was enriched in samples washed with 0.5 N ammonium formate (Table 18). 0.01 M phosphate buffer was a significant source of extracellular K (Table 20). Distilled water did not cause any more viability decrease than artificial seawater or cause any more trace element loss than ammonium formate or phosphate buffer (Table 20). Moreover, ultrapure water does not contribute any chemical contamination

to the sample. Accordingly, mechanizing Jones' (1977a) wash procedure and substituting ultrapure water for 0.5 N ammonium formate gave me a standardized and acceptable wash procedure.

One of the most controversial aspects of elemental analysis of biological tissue was the preparation of tissue for analysis (Gorsuch, 1966). Grant (1969) indicated that much of the total experimental error was introduced while preparing biological samples for elemental analysis. My early C, H, N data (Tables 12 and 13) substantiated Bowen's (1965) observation that meaningful data could only be obtained from homogeneous samples. Grant (1969) demonstrated that sampling error was reduced when dried tissue was ground to a fine powder. Steyn (1959) concluded that hand grinding with an agate mortar and pestle contributed less contamination than any other grinding technique. I adopted Steyn's technique for use in this investigation.

Procedures recommended for ashing biological materials included: dry ashing at 400-500 C (Baker, et al., 1964); Carpenter, 1964), low temperature ashing in plasma furnace (Riley and Roth, 1971; Walsh, et al., 1976), and acid digestion (Thiers, 1957; Gorsuch, 1970; Anderson, 1972; Abu-samra et al., 1975). Thiers (1957) discussed the relative advantage and disadvantages of each technique.

Grant (1969) demonstrated that Mn adsorbed to silica during dry ashing. Gorsuch (1966) reported significant losses of Sb, Zn and Pb during dry oxidation. Koirtyshann and Hopkins (1976) noted that Strohal had observed losses of Ce, Co, Mn, Pr, Ru and Zn from mollusc samples at temperatures as

low as 110 C. However, they could not detect any Zn, Fe, Cr, or Cd losses due to volatilization at 500 C. Koirtyshann and Hopkins reported that loss due to adsorption to the crucible surface was as high as 42 %. Professor C.L. Grant (personal communication) considered the sample container interaction during the charring process to be the primary source of trace element loss due to adsorption. He recommended isolating the sample from the crucible by wrapping the sample in an ashless filter such as Whatman #42.

Thiers (1957) indicated that Al, Cu and Sn tended to form insoluble complexes during dry ashing. Baker and Smith (1974) found Cu, Zn and Fe in insoluble complexes after dry ashing. I obtained insoluble complexes consisting of Na, Mg, Si, P, O, Fe and K (Fig. 8). Tsutsumi, et al. (1976) recommended post-treatment with an $HClO_4$-HNO_3 mixture. Fowler and Oregioni (1976) adopted a similar approach. The U.S. Environmental Protection Agency (1976) recommended using HNO_3 digestion followed by redissolution in HCl. Everson (1975) recommended digesting samples in volumetric flasks to eliminate metal adsorption to container walls as a source of error. However, the U.S. Environmental Protection Agency (1976) considered it better practice to filter the digested sample, rinsing the digestion beaker with ultra-pure water. Having been retained by the filter, insoluble complexes would not contribute to sample inhomgeneity and would not clog an AAS nebulizer orifice. I adopted my procedure taking these arguments into consideration.

After having standardized each successive process

from growth medium population through sample preparation, how precisely could I determine the elemental composition of P. cuprodurans, and how did this analyses compare with the precision obtained by other investigators analyzing biological material?

Carpenter (1964) determined the variability of repeated analysis of an individual sample of leaf tissue variability among subsamples of a single composite sample, and variability among samples (Table 60). My repeated analyses (Table 21) were more precise than Carpenter's. I obtained intraexperimental variations (Tables 48-55) of the same magnitude as Carpenter's, but had substantially greater interexperimental variation. Carpenter analyzed different leaf samples harvested from a particular tree making the comparison less direct.

Riley and Roth (1971) determined the precision of analysis for 14 elements in a composite ashed phytoplankton sample (Table 61). Again, their variation among subsamples was approximately of the same magnitude as mine. Murray (1973) analyzed A. marinus and determined intraexperimental variation of essentially the same magnitude. Murray obtained more precise Fe data. My interexperimental data (Table 57) for Mg and K were less variable, data for Na and Cu were equally variable, and data for Ca, Fe and Zn were more variable than Murray's (Table 62).

To assess the accuracy of the data, I compared my A. marinus, P. cuprodurans and E. coli data with Jones et al. (1977b) (Table 63). The data were not directly comparable,

Table 60. Statistical summary showing coefficients of variation of analytical results between replicate analysis of the same sample among 21 subsamples of a single composite sample and among different samples[1].

Element	Replicate Analyses of Same Sample			Analyses of Subsamples		Analyses of Different Samples			
	Replicates	[2]$\overline{ppm}$	CV %	$\overline{ppm}$	CV %	Samples	Replicates	$\overline{ppm}$	CV %
Al	5	51.6	4.44	59.0	13.7	100	4	48.0	6.4
Mn	5	29.4	13.9	27.7	15.4	77	4	86.4	9.3
Mo	5	1.75	30.6	1.44	26.9	100	4	2.3	13.3
Ca	5	.310%	1.6	.302%	2.38	100	4	.396%	5.4
P	5	.204%	1.9	.194%	3.02	100	4	.189%	6.5
Mg	5	.101%	2.7	.101%	3.12	100	4	.177%	6.7
Zn	5	18.8	7.98	18.4	7.08	100	4	20.78	20.6
Cu	5	20.4	37.6	17.8	14.9	80	4	13.82	63.3
Fe	5	86.8	4.5	93.0	8.85	100	4	43.46	26.5
B	5	6.54	17.8	6.1	22.9	93	4	34.66	6.03

Notes:

1) From Carpenter (1964)

2) $\overline{ppm}$: mean p.p.m. dry weight

Table 61. Analytical data and coefficients of variation for spectrographic determination of trace elements in a composite phytoplankton ash[1].

Element	Average Concentration in Ash (p.p.m.)	C.V. (%)
Ag	141	14
Al	142	9
Ba	264	10
Be	18	12
Mn	129	12
Ni	48	10
Pb	162	16
Cr	50	14
Cu	38	9
Sn	36	14
Sr	74	12
Ti	208	12
V	35	13
Zn	300	13

Note:

1) From Riley and Roth (1971)

Table 62. Analytical variation for composition of A. marinus among four subsamples from the same batch and among eight individual batches [1].

Element	Intra-Batch Variation		Inter-Batch Variation	
	[2] ppm	CV %	ppm	CV %
Cu	24.3	9.55	14.2	27.5
Pb	7.65	43.3	9.65	32.5
Cd	2.62	24.0	2.80	26.6
Zn	128.	12.3	122.	22.5
Ni	0.55	18.2	1.81	46.3
Fe	271	2.70	257.	22.2
Mn	1.75	16.5	1.72	26.0
Ca	104.	7.26	789.	49.4
Mg	1053	14.0	1580	46.9
Ca	104.	7.26	789.	49.4
Mg	1053	14.0	1580	46.9
Na	15000	11.7	5600	40.1
K	7020	9.61	660	54.1

Notes:

1) From Murray (1973)

2) ppm dry weight

Table 63. Comparison between [1]present analyses and [2]previous analyses of *E. coli*, *A. marinus* and *P. cuprodurans*.

Element	Organism					
	E. coli		*A. marinus*		*P. cuprodurans*	
	[3,4]Present Analyses	[5]Previous Analyses	[6]Present Analyses	[5]Previous Analyses	[7]Present Analyses	[5]Previous Analyses
Mg	1250	1351	2990	1440	4000	292
Na	2160	3341	11500	5600	2800	255
Ca	2340	42	1360	790	2600	272
K	4490	1789	1410	700	2150	36
Fe	140	111	333	257	170	422
Zn	46.3	76	154	122	97	112
Cu	49.6	11	10	14	27	19

Notes:

1) Data from Tables 22, 34, and 59
2) Data from Jones et al. (1977b)
3) Figures are ppm dry weight
4) Cells were grown in modified 2216E; harvested without washing
5) Cells were grown in tryptone-yeast extract basal seawater medium; washed three times in 0.5 N ammonium formate
6) Cells were grown in modified 2216E; washed once in 0.5 N ammonium formate.
7) Cells were gorwn in modified 2216E; washed three times in Q2W.

since I grew and harvested the cultures differently than Jones did. However, for all three cultures, Jones et al. (1977a) and I obtained similar Fe, Zn and Cu concentrations. These elements were not affected appreciably by washing (Table 34). Substantial discrepancies existed among the major cation concentrations. This paralleled the response to washing (Table 34). Bowen (1966) listed 115,000 ppm K, 5100 ppm Ca, 4600 ppm Na, and 7000 ppm Mg representative values in bacteria. Although my K concentration was two orders of magnitude lower than Bowen's, my Na, Ca, and Mg were in reasonable accord with Bowen's estimates. Tacas, Matula and Mac Leod (1964) reported intra-cellular Na and K concentrations in Alteromonas marinopraescens ranging from 4.75 - 1024 mM and 19.4 - 28.6 mM, respectively depending on Na and K concentrations in the growth medium. These values compared well with those in Table 57. Thompson and Mac Leod (1973) observed substantial losses of K and Na during washing. They suggested that the enzyme system for cation uptake had been insulted sufficiently by the wash procedure to be unable to balance cation loss which proceeded at a constant rate.

The data base on which I based the preceeding discussion was small. The discrepancies between my data and Jones' (1977b) reflected the need to apply standardized procedures to each contributory process in cultivating and analyzing bacteria. Compositional variations in the medium appeared to induce compositional variations in the bacteria (Jones et al., 1976, 1977c), but the changes were complex and required further investigation.

I believed that much of the variation between my data and Jones' (1977b) could be attributed to differences in post-cultivation processing. I concluded that at the present state-of-the-art, it would be meaningless to discuss absolute elemental compositions for bacteria or even higher organisms. Instead, I propose that the data base be broadened, first for a single species, then for a variety of species. Data were not sufficiently precise to resolve the effects of minor environmental changes, but were precise enough to reflect environmental changes. I would include physical, nutrient, and heavy metal stresses in the latter category. Minor and trace elements in the ash fraction were better suited than major consitutuents such as Na, K, Ca, and Mg for interexperimental comparisons. The elements present at concentrations $\leq$ 200 ppm were less susceptible to unintentionally introduced experimental variation than those present at higher concentrations and should provide the key to evaluating environmental and genetic factors controlling the elemental composition of bacteria.

Whether elemental composition was determined genetically, environmentally or both remained a moot issue. Although scattered evidence indicated that trace metal concentrations were species specific (Stiles, 1961; Cowgill and Burns, 1975; Sokolova and Chernitskii, 1976), most reports did not find this specificity (Riley and Roth, 1971; Zook et al., 1976). Moreover, a number of investigators have presented eloquent arguments supporting the theory that the geochemistry of trace metals at biosphere-hydrosphere interfaces was essentially

the same as it was at lithosphere-hydrosphere interfaces (Ermolenko, 1966; Diamond and Wright, 1969; Hodges, 1973; Wakatsuki, et al., 1974; Jernelöv and Martin, 1975). Apparent species specificity was probably a reflection of the physiological state of the organism as it affected the charge configuration of the cell envelope. Obviously, in this respect, the extent of metal uptake by a particular species would be under genetic control. Resolution of this problem would be possible only after a data base of statistically comparable elemental data was obtained.

There remains a great deal of useful information to be obtained regarding the composition of living organisms. I do not think that it will be practicable in the near future to harvest mixed populations from natural environments and make assessments regarding the biogeochemistry of the environment based on multielemental analysis of the sampled population. I do think, however, that we can apply this line of investigation to answering questions regarding the function and composition of biological membranes and their roles in metal transformations and biogeochemical cycling.

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